Optimization of Water Use Efficiency in Tomato (*Solanum lycopersicon* L.) by Transposition of an *LeNCED1* Transgene

By

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List of abbreviations

A_n	Assimilation
ABA	Abscisic acid
Ac element	Transposon; Activator element
ANOVA	Analysis of variance
AS-AO	ABA specific aldehyde oxidase
BCH	Beta carotene hydroxylase
CaMV	Cauliflower mosaic virus
CCD	Carotenoid cleavage dioxidase
cDNA	Complementary DNA
CIRAS	Compound infra-red analyser system
cM	Centi-Morgan
cm	Centimeter
cv.	Cultivar
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribo nucleotide triphsphate
Ds element	Transposon; Dissociation element
DW	Dry weight
et al.	and others
e.g.	For example
g	Grams
GA	Gibberellic acid
g_s	Stomatal conductance
i.e.	That is
Kg	Kilogram
L	Litre
LA	Leaf area (cm^2)
LER	Leaf expansion rate
LSD	Least significant difference
mRNA	Messenger ribonucleic acid
µg	Microgram
µl	Microlitre
m	Meter
ml	millilitre
Mill.	Miller
min	Minutes

NAR	Net assimilation rate
Nz	Norflurazon
NCED	9-cis-epoxy carotenoid dioxygenase
NCEI	9-cis-epoxy carotenoids forming isomerase
nptII	Neomycine phosphotransferase II
NSY	Neoxanthin synthase
ORF	Open reading frame
%	Percent
P	Probability
PCR	Polymerase chain reaction
pH	Hydrogen ion potential
Q-PCR	Quantitative pcr
QTL	Quantitative trait loci
RGR	Relative growth rate ($g\ g^{-1}\ day^{-1}$)
SED	Standard error of difference of means
T_1	Generation resulting from selfing of T0-generation individuals
T_2	Generation resulting from selfing of T_1-generation individuals
$Tm2^a$	Tomato wild type containing TMV resistance gene
T-DNA	Ds element containing transfer DNA
T-DNA-Ds	Ds element linked to T-DNA
Tr-Ds	Transposed Ds element
WT	Wild type
WUE_p	Plant water use efficiency

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Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged

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Summary

The 9-*cis*-epoxycarotenoid-dioxygenase (NCED) is a key regulatory enzyme in ABA biosynthesis in plants and its overexpression increases ABA levels that can increase water use efficiency (WUE). The use of the 'super promoter' (sp) to drive an *LeNCED1* transgene in tomato greatly improved WUE without affecting long-term plant growth but caused delayed seed germination and reduced rates of seedling establishment. The first aim of the present study was to generate useful, novel variation in NCED transgene expression by allowing an *LeNCED1* transgene, driven by a histone H2A promoter (pH2A), to transpose to new positions in the genome.

A stabilized activator element linked to a GUS marker gene (*sAc*) was used to allow transposition of an engineered dissociation element (*Ds*) containing the *LeNCED1* transgene of format Ds1::pH2A::LeNCED1::Ds2 (T-DNA-Ds). In F_1 (*sAc* × *Ds*) plants transposition was demonstrated by detection of empty donor site through PCR. The F_2 generation was screened for stable integration of the *Ds* element and reduced g_s. Three F_3 families, namely $59F_3$, $102F_3$ and $116F_3$, were investigated: they showed multiple stable *Ds* transposition events, had low g_s, and a range of growth rates. Genotype $102F_5$ had wild-type seed germination, higher bulk leaf ABA and xylem sap ABA and 60% higher gravimetric WUE (WUE_p).

However, $102F_5$ accumulated 32% less above ground dry weight than wild type under well-watered conditions 56 days after germination. The *sAc* and *Ds* approach allowed production of T-DNA-Ds and nptII kanamycin free $102F_5$ line which retained transposed *Ds* (*Tr-Ds*) elements. These progeny were cross pollinated with *sAc* to reactivate the transposition of the *LeNCED1* transgene and should be an excellent material for generating further variation in ABA content.

The second aim was to overcome the seed dormancy in high ABA, genotypes such as sp5 and sp12, by testing novel hydroxamic acid NCED inhibitors for their ability to stimulate germination and seedling establishment. Improvement in seed germination in sp12 was observed with two hydroxamic acid compounds, and an increase in seedling growth rate was also observed, although this was not statistically significant.

Chapter-1

General Introduction

1.1 Water, an essential element for life

Water is essential for life and without availability of fresh water it is impossible for terrestrial organisms to survive. On the land, plants typically require more water than animals due to higher water losses through transpiration, and hence water availability is one of the most important factors in determining plant growth, development and yield. Although more than three quarters of our planet is covered with water only 2.5% of the total is fresh water, and only a small proportion of this is available for agriculture, with the majority being locked up in glaciers and permanent snow cover (Figure1.1).

The first major civilisations are believed to have developed on the basis of irrigated agriculture in the near east regions in particular Egypt and Mesopotamia in about 5000 B.C. People from these civilisations understood the importance of fresh water in successful crop production. It is estimated that water conservation has been practiced by farmers in the drier environments for more than 2000 years by building underground water channels known as '*Qanats*'in Iran, '*Falaj*' in Arabian Peninsula and '*Kareezes*' in Afghanistan (English, 1968).

1.1.1 Role of climate change in water availability for crop production

The global hydrological cycle (a continuous flow of water on, below or above the surface of Earth) annually generates enough fresh water to sustain more than 7 billion people in the world but the distribution of this water is not even in time or space

and it is not all accessible for human use (Figure1.1). Only approximately 31% of the total rain water can be used because 50% is wasted in floods and 19% is too remote to access (Postel, 2000). Even by building a large number of dams and reservoirs to store the flood waters, it is estimated that no more than 10% of this rainfall can be stored.

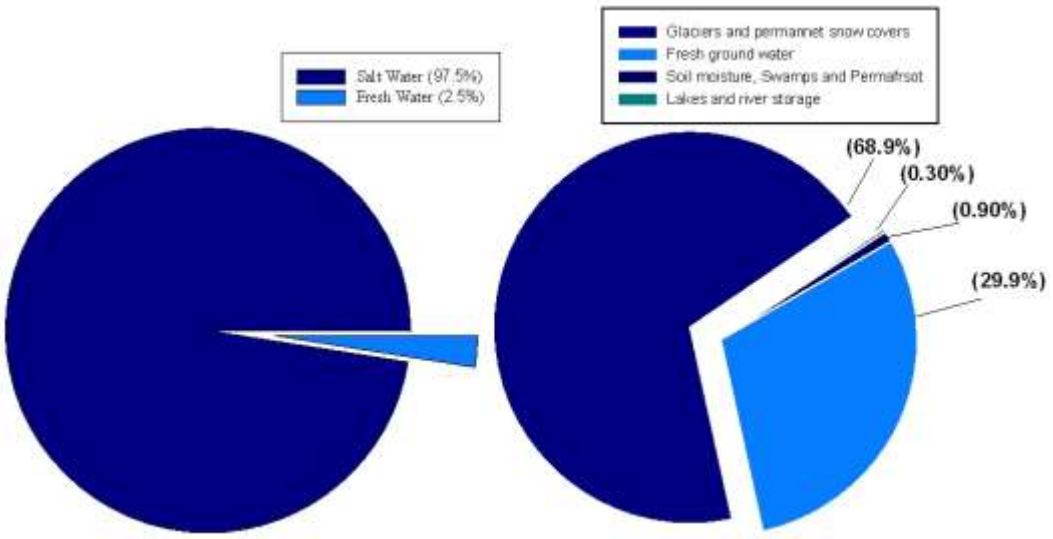

Figure 1.1. Global partitioning of water. Left, division between fresh and salt water; right, distribution of total fresh water (Maurits La Riviere, 1989).

Solar energy drives the evaporation of water of which 86% occurs from oceans and 14% from land, 24% of total precipitation falls on the land which amounts to approximately 1.2×10^{14} m^3 $year^{-1}$ (Shiklomanov, 1993). The predicted rise in temperature due to increasing greenhouse gases may result in increased water flux through the water cycle resulting in more severe weather events in certain parts of the world. But high temperature might also result in less snow deposits and melting of glaciers which supply a bulk of the fresh water in the rivers and aquifers during the dry season, though this issue has been found controversial (Bagla, 2009).

As predicted in the International Panel on Climate Change's (Sanderson et al.) 4th report, a rise in temperature in most parts of the world might affect plant growth. As yield through higher evapotranspiration, as the plant reproductive stages are particularly sensitive to heat and drought stress e.g. through pollen degeneration, reduced Harvest Index (HI) (Li et al., 2010), higher evapotranspiration rate would result in greater soil water deficits. However, in some cool temperate regions increased temperature might increase crop yield.

However, the limited ability of climate models to make long-term forecasts, because of uncertainty and the involvement of a range of variables and their interactions with each other, makes it extremely difficult to exactly predict the role of changing climate on future crop growth and yield under field conditions. This makes it extremely difficult for plant breeders to produce varieties suited to our future climates.

1.1.2 Water bankruptcy: Implications for the Future

A growing scarcity of fresh water due to an increase in demand and climate change is now evident in many parts of the world. The expected population increase by 2050 is projected to be 30-35%; this is predicted to leave the vast majority of the developing world population in Sub-Saharan Africa and South Asia under severe water stress conditions (Postel, 2000). According to an estimation the shortfall of fresh water supply by 2050 will be 6000 $km^3 year^{-1}$ which is equivalent to the annual flow of 6 Nile rivers (Cribb, 2010).

Water scarcity can result in declining food production and increased food prices. According to International Food Policy Research Institute's report on 'Global water outlook to 2025' cereal food prices may be doubled under a business as usual scenario

which could cause further famine and loss of lives in developing countries especially in Asia and Africa. This report suggested that improving water conservation and water use efficiency can be a solution to avoid future problems arising from water scarcity. The extent to which the scarcity of water could lead to worldwide famine due to food shortages was so grave that the Secretary General on United Nations in April 2000 emphasized the need for a 'Blue Revolution' in agriculture to produce more "crop per drop" (Pennisi, 2008).

Although some parts of Europe have ample annual precipitation, its distribution is not uniform throughout the crop growing period. Despite having a seemingly wet climate some parts of the UK are experiencing water shortages. The prediction made by the UK climate impact programme (UKCIP), 2009, was that there could be up to 25% less precipitation during the summer season in Southern England by 2080 (medium carbon emissions scenario, 50% probability). This would significantly reduce the amount of 'green' water (water present in soil) and 'blue' water (water available in rivers and aquifers), which could reduce crop production under the higher evaporative demand due to predicted elevated temperature (+2.7 to 4.1^{o}C), reduced relative humidity (-9%) and increased sunlight (+20 Wm^{-2}) due to reduced cloud cover during the day (UKCIP, 2009). Under such circumstances it might not be economical to grow crops under rain-fed conditions, where the yield will drop below the profitability threshold. Further, under such conditions, more pumping of ground water for irrigation could lower the water table exacerbating the problem of low water availability.

Since water is arguably the most important factor in limiting crop production there is a pressing need to improve the yields of crops in water-limiting environments and to use water resources in a more sustainable and efficient manner.

1.2 Tackling the problem of acute water shortages

1.2.1 Soil water conservation through improved tillage

Nearly every country in the world faces water shortages at some point during the year (Gleick, 1993), and it was estimated by Falkenmark and Lindh (1993) that approximately 80 countries will face serious water shortages in the near future (Falkenmark and Lindh, 1993). The present worldwide analysis has revealed that nearly 90 countries in the world are suffering from some degree of drought (Yuen, 2011)

Soil water conservation can be achieved by employing various on-farm techniques. The term tillage encompasses a variety of activities and from one farm to another can vary greatly, for instance it can be simply ploughing the field or sophisticated use of technology e.g. laser levelling aimed at efficient use of available irrigation water by reducing the run-off. The major unproductive water losses from the field are by direct evaporation of water from the soil surface and vertical drainage of water beyond the root zone (Batchelor et al., 2002). Tillage systems can affect the soil physical and chemical environment and improved soil tilling techniques can improve the capture of soil water by avoiding soil compaction, increasing the root depth and by enhancing soil aeration and soil microbial activity (Kladivko, 2001).

1.2.2 Efficient irrigation techniques

During the rainy season the excess rain water could be stored in reservoirs to be used during the dry time of the year. Similarly, construction of water channels to avoid seepage and use of efficient irrigation system such as drip/trickle irrigation may waste less water than other systems such as sprinkler or flood irrigation.

1.2.3 Breeding water efficient crops

Water can also be saved by producing crop varieties with high water use efficiency using the existing gene pool or by manipulation of existing plant strategies to combat drought under natural habitat. Being sessile, plants cannot acquire water from distant areas where it is abundantly available. In order to survive under water limited environments, plants have evolved strategies through evolution and natural selection. On the basis of their ability to survive under stress conditions, adaptive mechanisms have been categorised into three distinct categories.

1.2.3.1 Drought escape

This mechanism has been developed to escape the intense drought period. Such plants tend to complete their life cycle rather quickly through rapid metabolic and growth rate (McKay et al., 2003). It has been suggested that these plants have higher g_s and photosynthetic ability compared to other C3 plants (Mooney et al., 1976). There are however, implications as these plants have a relatively low yield potential as they cannot make a full use of seasonal solar radiation (short growth period and allocation of resources to reproductive parts rather than vegetative organs such as leaves) to produce maximum photosynthates. According to Hay (1999),

$$Y = RAD \times \%RI \times RUE \times HI \quad \dashrightarrow \quad \text{Equation -1}$$

In equation-1 ‘Y’ is the grain yield potential, RAD is total amount of solar radiation received, %RI is intercepted radiation by canopy, RUE is radiation use efficiency and HI is harvest index (Hay, 1999). This equation shows that as the amount of radiation

received is decreased total yield is also reduced due to lower accumulation of photosynthates.

1.2.3.2 Dehydration tolerance

This mechanism enables the plant to tolerate conditions of cellular dehydration, so that they can rapidly recover when water becomes available. The Resurrection Plant is an extreme example of dehydration tolerance (Bartels and Salamini, 2001) where less than 2% relative water content in the leaves can be tolerated (Bartels et al., 1990). Several changes at the metabolic, transcriptomic and proteomic level occur during dehydration, ensuring that the tissues become dehydration tolerant. Some other salient features adapted by these plants include repair of structural damage to subcellular components due to reactive oxygen species (ROS) and increased presence of protective Late Embryogenesis Abundant proteins (LEAs).

1.2.3.3 Dehydration avoidance

This mechanism allows a plant to sustain an acceptable plant water status (cellular hydration) when the environmental availability of water is reduced. These plants can survive the scarce supply of water without exposing the vital cellular components required for plant growth and development to damaging cellular water deficits. These mechanisms include reduction in water loss through decreasing $g_{s,}$ deposition of cuticular wax and leaf rolling, enhanced root growth to improve water uptake and osmotic adjustment to maintain turgor pressure (Ψ_p) under low water potential (Ψ_l) conditions.

The mechanism of dehydration avoidance has been well studied on a genetic and molecular basis. Further, most plant breeders are trying to produce dehydration avoidance varieties with the ability to survive for longer and are able to produce more biomass and yield under drought conditions.

In the present study, one of the dehydration avoidance approaches was used to breed plants with drought resistance through manipulation of ABA which can have a direct influence on water loss, water uptake and plant water use efficiency (WUE_p).

1.3 Water use efficiency in plants

Plant WUE can have different meanings to different disciplines and it depends on the scale of measurement and the unit of exchange, for instance, a plant physiologist might be interested in instantaneous WUE (WUE_i) (measured at a leaf scale) which can be described as moles of carbon assimilation per mole of water being transpired (Farquhar and Richards, 1984) as measured by leaf gas exchange. For an agronomist it is the amount of dry matter produced per unit water supplied to, or used by, the crop (Condon et al., 2004).

So

$$WUE = \frac{\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad}{\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad}$$

or

$$WUE = DM/T \quad \dashrightarrow \qquad \text{Equation -2}$$

where ‘DM’ is the dry matter produced and ‘T’ total seasonal transpiration. But under field conditions water is also lost from the exposed surfaces in the field as evaporation, hence, equation-2 can be rewritten as;

WUE = ——— ------→ Equation -3

where E_s is the evaporation from the soil surface. Equation-3 gives a better picture of WUE as it highlights the importance of evaporation as a source of water loss even though it is not being used by the plants.

Condon et al. suggested another framework to define WUE for farmers and agronomists (Condon et al., 2004), i.e.

Yield = ET × — × WUE × HI ---------→ Equation -4

In this equation ET is evapotranspiration, T is transpiration, 'T/ET' is the proportion of water lost through transpiration, HI is the ratio of grain yield to the total aboveground dry biomass, and WUE is the biomass gain per mm of water transpired. The model presented in equation-4 has been useful for agronomists as it clearly demonstrates the factors which contribute towards increasing yield in the context of WUE. It also highlights the need to reduce E_s (i.e. increase T/ET) which can be achieved by growing crops which establish faster to cover soil or by soil mulching. WUE_p can be improved as discussed below and HI can be increased by changing the source to sink ratios with increased biomass allocation towards, for example, grain filling or fruit formation.

1.3.1 Factors contributing towards improving WUE_p

Various plant and environmental factors contribute towards WUE_p, some of the most important factors include CO_2 assimilation rate (e.g. in C_3 and C_4 plants), VPD and stomatal behaviour.

1.3.1.1 Vapour pressure deficit and concentration of CO_2

In a plant, the rate of transpiration is dependent on the vapour pressure deficit (VPD) between leaf and air surrounding it. As the VPD increases the rate of transpiration should increase as well. But the flow of water molecules from within the intercellular spaces to the outside environment is restricted by the resistance to diffusion from stomata and leaf boundary layer (Bierhuizen and Slatyer, 1965). This is shown as follows;

$$T = (w_i - w_a)/(r_s + r_a) \quad \text{Equation-5}$$

Here in equation-5 'w_i' and 'w_a' represent the partial pressures of water vapour inside the mesophyll tissue air spaces and outside in the atmosphere, respectively, while 'r_s' shows stomatal resistance and 'r_a' indicates the leaf boundary resistance (detail present in section 1.4). Therefore, from equation-5, it can be deduced that increased stomatal resistance (r_s) will reduce water loss, but this will also reduce the rate of CO_2 fixation by increased resistance to CO_2 (detail present in section 1.4).

However, crops which could maintain a low cellular CO_2 can prove useful to increase WUE_p, for instance C4 plants (4-carbon dicarboxylic acid) usually have lower mesophyll resistance and low CO_2 compensation point compared to plants having C3 metabolism. Hence, in C4 plants net resistance to CO_2 is lower than the resistance to escaping water molecule resulting in improved WUE_p (Slatyer, 1970). It is also important to mention that the plants grown under humid and temperate regions will have higher WUE_p than the plants grown in arid and semi arid conditions (Cooper et al., 1987) due to lower VPD.

1.3.2 Soil plant atmosphere continuum (SPAC)

The concept of SPAC provides a framework to study plant water use. It recognises that the soil, plant and atmosphere constitutes a dynamic system which is physically integrated, where the flow of water and energy consumed during its flow occurs simultaneously and independently in the form of a chain. SPAC is controlled by the hydraulic resistance of soil, root, stem, leaf, stomata, cuticle and boundary layer in the continuum (Figure1.2).

1.3.2.1 The scale of water transfer

Water is lost continuously from leaves and it has been estimated that terrestrial plants transpire up to 40,000 km^3 $year^{-1}$ which constitutes approximately 70% of total evapotranspiration from the land (Brodribb et al., 2010). Stomata are the main escape route of this water, but the main function of stomata is to allow uptake of carbon dioxide (CO_2) for carboxylation during photosynthesis. A relatively small amount of water is also lost when stomata remain partially open for a continuous flow of oxygen during respiration at night.

1.3.2.2 Factors that affect water flows

Crop evapotranspiration (ET) is influenced by both environment and the crop. The crop factors include: the dynamics of leaf area, i.e. its development and senescence, resistance to water flux which is developed in the SPAC and water capture from the soil. Water movement through the SPAC is regulated by the Ψ_l gradient because water flows from higher to lower Ψ_l. Pure water has a potential value of zero and it decreases as the

solute concentration increases. The Ψ_l also depends on the matric potential (Ψ_m) and turgor potential (Ψ_p) of the plant cells.

The energy input driving the water flows through SPAC is mainly solar radiation which is absorbed by the canopy. Vapour pressure deficit, air temperature and wind speed also play crucial role in plant water flux; leaf water status varies considerably during the same day and even a passing cloud can dramatically influence the water flux through a plant. The leaf Ψ_l equates to the soil at dawn, since the two values equilibrate overnight, but often will decrease from sunrise to the midday because atmospheric demand for water outstrips water supply from the soil. So, at dawn there is minimum stress on plants, while at midday the plant is under maximum stress for a given soil water potential.

1.3.2.3 Resistance to hydraulic flux in the plants

Plants species vary greatly in the resistance to water movement inside different tissues and water flux through the plant faces resistance on each level starting from its absorption in the rhizosphere to its diffusion to the atmosphere through the stomatal pores. In other words the movement of water obeys Ohm's law by analogy (current equals driving force divided by the resistance across the circuit) shown in Figure 1.2. Under steady state, flow through each segment of SPAC can be described below;

, , represent the water potential of the soil, root, leaf, and air whereas, , , , are the resistance from soil matric, roots, xylem, stomata and atmosphere, respectively (Blum, 2011). This equation shows that water in a plant flows

due to the difference in Ψ_l in different plant tissues against the resistance instigated by the tissues or surrounding environment. Further, when present in a series (like in a plant) all these resistances are added together.

In a well hydrated plant the minimum hydraulic resistance is posed by stem and maximum hydraulic resistance is experienced in leaves, and the key points of resistance are considered below in the following sections.

1.3.2.4 Root resistance

The root system is the most crucial tissue in plants which supplies water to a plant under a high demand. Root hydraulic resistance is inversely proportional to the root length density. As the root length density increases the contact points between soil water and root axes increase. Studies with plants expressing high endogenous ABA revealed that ABA improved root hydraulic conductivity (Thompson et al., 2007). ABA has also been found to increase the cell to cell radial hydraulic flux (Parent et al., 2009). It has been suggested that ABA increases aquaporin gene expression and may interact with aquaporins at the plasmalemma to increase the root hydraulic conductivity (Kaldenhoff et al., 2008).

1.3.2.5 Stem resistance

The axial (longitudinal) hydraulic resistance in the stem is low compared to that in stomata, leaf and roots. The conduits of xylem have evolved an efficient hydraulic flow system to allow minimum resistance during high transpirational demand and ascent of water to long distances particularly in the stem. The water molecule contains a dipole and the partially negatively charged oxygen atom forms a hydrogen bond with a

partially positively charged hydrogen atom of another molecule. This keeps water in liquid phase and also provides surface tension (caused by intermolecular forces). This force allows plants to pull water from roots to the leaves through the xylem. Though, under drought and high ET demand, the xylem vessels can be prone to embolism (Sperry, 2003) which can cause a significant reduction in hydraulic conductivity.

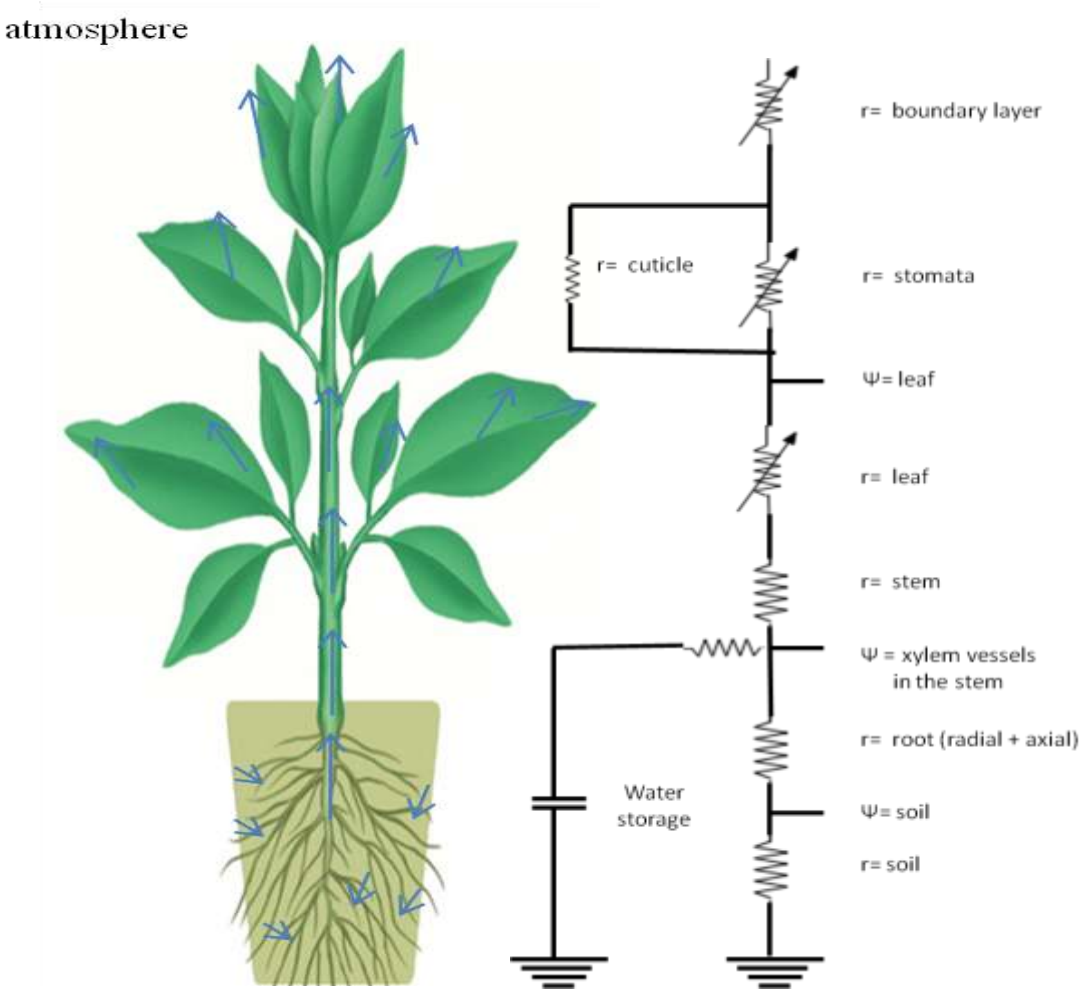

Figure 1.2. A schematic representation of soil plant atmosphere continuum analogised with Ohms' law. The battery icon shows the stored water; resistance icons show the resistance to flow of water caused by various plant organs; resistance icon with an arrow indicates variable resistance; r shows the hydraulic resistance and Ψ indicates the water potential. The blue arrows on the plant show the flow of water due to gradient in water potential and transpiration pull. This figure was modified from (Blum, 2011)

1.3.2.6 Stomatal resistance

Stomata can be regarded as under the direct influence of chemical and hydraulic signals. Stomatal movements are extremely sensitive to the environmental conditions e.g. CO_2 concentration, VPD and light. Regulated turgor changes in guard cells are

responsible for the stomatal movements. Stomata can sense the long-distance chemical signals from the root in the form of pH, ABA and cytokinin which can influence the stomatal resistance. Guard cells dynamically regulate the size of stomatal aperture to allow photosynthesis and avoid excessive loss of water from mesophyll tissues under the influence of high VPD (Acharya and Assmann, 2009).

A great deal of work has been carried out to understand the physiology of the guard cells in the last three decades which revealed that cytosolic Ca^{+2} activates the plasma membrane anion channels (Schroeder and Hagiwara, 1989); two pore K^+ (Gobert et al.) channels in *A.thaliana* also open due to changes in cytoplasmic Ca^{+2} and cytoplasmic pH (Gobert et al., 2007). Similarly the cytosolic Ca^{+2} is responsible for the down regulation of proton pumps in Fava bean (Kinoshita et al., 1995) and inward rectifying K^+ channels (Schroeder and Hagiwara, 1989). This causes reduction in the turgor of the guard cells, hence stomatal closure. Stomata are therefore complex resistors in the SPAC which can respond to many environmental signals.

1.3.3 Assimilate partitioning

Crops grown under reduced water supply tend to increase their root: shoot ratio, and if the WUE_p is calculated using above-ground parts excluding the root biomass, this results in artificially low WUE_p. However, if the crop varieties could be produced with more photosynthates allocated towards economically important plant parts such as grains, fruits, stems (potato) and roots (carrots, radish, turnip) and leaves (spinach, salad, cabbage), under reduced water regimes, such crops will have higher HI and possibly higher economic WUE.

1.3.4 Breeding for high WUE by reducing stomatal conductance

Stomatal conductance is reciprocal of stomatal resistance and it has curvilinear relationship with the amount of CO_2 being fixed and water lost through stomata, hence it is an ideal parameter to measure water loss compared to rate of assimilation(A_n), as water loss (g_s) can be easily measured and compared to A_n (Araus et al., 2002). Leaf g_s can be defined as the proportionality constant between the rate of transpiration and VPD between the inside and outside of the leaf (Sims and Pearcy, 1989). Leaf g_s can be measured at the leaf level by using a Porometer or Infra Red Gas Analyser (IRGA).

At the leaf level, A_n is a function of g_s to CO_2, C_i (Earl) and C_a (CO_2 concentration around the leaf) hence, it can be expressed as;

$$WUE_i = 0.6\, C_a\, (1- C_i/C_a)/VPD \quad \text{------►} \quad \text{Equation-7}$$

In equation-7, 0.6 refers to a constant of relative diffusive ability of CO_2 and water inside the leaf and VPD refers to water vapour pressure deficit from within the leaf to outer atmosphere, respectively. Equation-7 shows that the leaf level WUE can be improved by either decreasing the value of C_i/C_a, this will result in increased value of $1-C_i/C_a$ or by reducing the VPD, in other words, to reduce the driving force required for transpiration (Condon et al., 2004).

At lower values of g_s, the CO_2 assimilation increases linearly as g_s increases, but at higher values of g_s, A_n begins to saturate due to non-stomatal limitations e.g., saturation in regeneration of ribulose 1-5, bisphosphate, while transpiration continues to increase in a linear fashion. Thus as g_s increases beyond a certain point, WUE_i begins to decline (Figure 1.3). This illustrates how higher WUE_i can be achieved when g_s is lower than its potential maximum for a genotype, however, at too low g_s, assimilation of CO_2 is also low which can result in reduced growth (Blum, 2005), and again lower

WUE_i (Figure 1.3). Although plants with reduced g_s are assumed to result in lower crop production (Condon et al., 2002), Figure 1.3 also shows the possibility of greatly improving WUE_i with only a very small yield penalty, as at point 'X' CO_2 assimilation has almost been saturated but transpiration is still increasing in a near-linear fashion. The g_s is regulated by stomatal movement which is controlled by the phytohormone abscisic acid (ABA). ABA signalling is therefore an important target for breeding improvements in WUE.

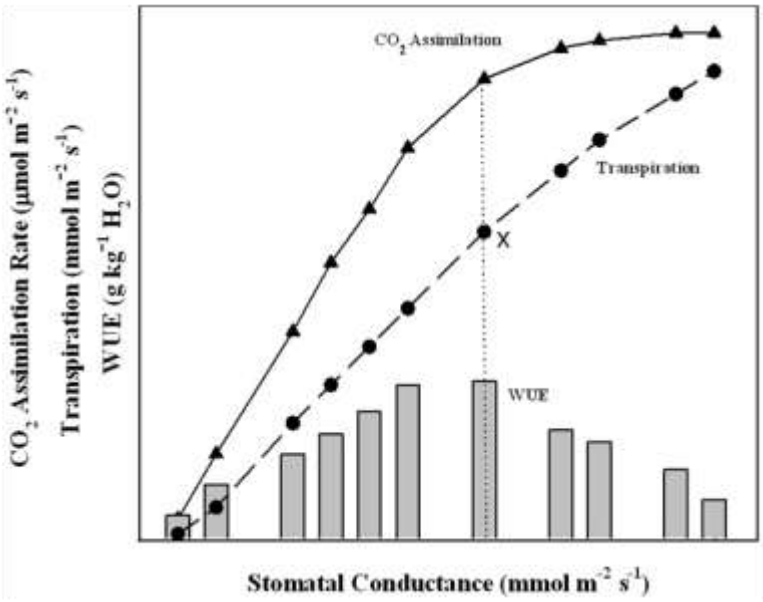

Figure1.3 An hypothetical illustration of relationship between g_s, CO_2 assimilation and WUE_i in plants. Triangles represent net CO_2 assimilation rate, whereas circles show transpiration and bars on x-axis indicate WUE_i. Point 'X' on the graph shows theoretically maximum WUE_i in plants. Modified from (Yoo, 2009, Regulation of Transpiration to Improve Crop Water Use).

1.4 Discovery of ABA

ABA has been known to plant scientists since the 1960s, initially to be called 'dormin' as it induces bud dormancy or 'abscissin' because it promoted leaf abscission (Ohkuma et al., 1963, Cornfort.Jw et al., 1966, Evans, 1966, Vanoverb.J et al., 1967). Later during the same decade this hormone was renamed as ABA (Addicott et al.,

1968). Since its discovery a great deal of work has been done on the chemistry and physiology of ABA which occurs universally in vascular plants.

ABA is involved in a diverse range of functions, but there are still some gaps in the understanding of the exact mechanisms of action in some plant functions. The salient features of ABA are described in the following sections.

1.4.1 ABA as a plant stress hormone

Plant roots are finely tuned and can sense very small changes in soil water status (Davies and Gowing, 1999) and, in order for plants to cope with drying soil, this signal has to reach the shoot, resulting in changes in the shoot response such as reduced g_s or leaf expansion as a consequence of accumulation of ABA in the xylem sap (Davies et al., 2000). Dehydration in plants results in a reduction of leaf Ψ_l which eventually causes a decline in Ψ_p and loss of Ψ_p is one of the most closely related signals for ABA biosynthesis (Creelman and Zeevaart, 1985). This chemical signal generated in the form of ABA is involved in the root-shoot signalling which can result in stomatal closure whose redistribution was thought to change due to pH changes in the xylem sap of a plant under drought stress (Tardieu, 1996, Sauter et al., 2002).

The regulation of ABA biosynthesis could be used to control drought resistance and can improve plant water use efficiency (Thompson et al., 2007, Pou et al., 2008, Liu et al., 2005).

1.4.2 ABA Biosynthesis

ABA is a member of metabolites known as isoprenoids, also called terpenoids. These are derived from building up units of a common isopentanyl C_5 precursor. The

isoprenoids are present in plastids, including carotenoids, originate from combination of IPP and DMAPP (dimethyl pyrophosphate) an isomer of IPP, which is synthesized from the mevalonic acid- (MVA) independent pathway known as the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Eisenreich et al., 2004).

In phytopathogenic fungi, ABA is thought to be synthesized by a direct pathway, where the C_{15} compound farnesyl pyrophosphate is used, but in plants, ABA is synthesized by an indirect approach where oxidative cleavage of xanthophyll precursors occurs. Xanthophylls are C_{40} oxygenated carotenoids, and ABA is produced from C_{15} intermediates which result from cleavage of xanthophylls (Zeevaart and Creelman, 1988).

ABA biosynthesis in plants has been extensively studied by using genetic approaches. Some of the maize viviparous mutants (*vp2,vp5 and vp9*) showed albino phenotype due to defective carotenoid biosynthesis (McCarty, 1995). ABA mutants in tobacco showed a phenotype where plants had severe epinasty; such phenotypes were found to have a defective xanthophyll cycle (Marin et al., 1996). This and other evidence suggested that ABA is derived from carotenoids. In experiments involving ^{14}C radiolabelled xanthoxin, it was revealed that this aldehyde was converted into ABA demonstrating that xanthoxin was an intermediate in the ABA biosynthesis pathway (Taylor and Burden, 1972). Further, wilted leaves of *Xanthium strumarium* were incubated with a mixture of $^{18}O_2$ and N_2, the results showed that only one atom of ^{18}O was incorporated into the ABA molecule, located in the carboxylic group. This result was unexpected, as it was believed that ABA was produced from a C_{15} precursor such as farnesyl sulphate, through the MVA (direct) pathway. In that case ^{18}O should have been incorporated into the 1′-OH and 4′-keto groups, but since these groups remained

unlabelled, it was suggested that these groups were provided by xanthoxin (a stored precursor) which exists with an oxygen atom already present at the 1′-OH and 4′- keto positions (Creelman and Zeevaart, 1984). This evidence showed that ABA biosynthesis occurs through carotenoid cleavage via the 'indirect' MEP pathway (Rohmer, 1999). The MEP pathway is localised in plastids in higher plants while the MVA pathway occurs within the cytoplasm (Lichtenthaler et al., 1997). A key step in the MEP pathway is when IPP and DMAP undergo head to tail condensation and produce geranylgeranyl diphosphate which is the substrate for phytoene synthase.

1.4.3 Phytoene synthase and desaturase

Production of phytoene is the first committed step in the biosynthesis of carotenoids; the enzyme catalysing this reaction is phytoene synthase (PSY). This is a key step in carotenoid biosynthesis which has been commercially exploited by over-accumulation of carotenoids in Golden Rice (Paine et al., 2005). Generally phytoene does not accumulate in plant tissues (Hirschberg, 2001) but it is considered to be an intermediate in the production of other carotenoids.

Phytoene desaturase converts phytoene to ζ-carotene by introduction of two *trans* double bonds. Both phytoene desaturase and ζ-carotene desaturase need plastid terminal oxidase and plastoquinone which act as electron acceptors (Hirschberg, 2001).

Plastid terminal oxidase was found to be an important component of the electron transport chain and for the respiratory activity within plastids (Carol and Kuntz, 2001). Plant mutants for plastid terminal oxidase showed accumulation of phytoene, carotenoid deficiency and an albino phenotype due to the resulting photobleaching of cotyledons (Carol et al., 1999). The phenotype of these plants was similar to the plants treated with

herbicide norflurazon which targets specifically phytoene desaturase (Busch et al., 2002).

In plants desaturation of carotenes is catalysed by two enzymes phytoene desaturase acting on phytoene and ζ-carotene desaturase which acts on ζ-carotene (Matthews et al., 2003). These desaturation steps create conjugated carbon double bonds that transform colourless phytoene to red lycopene.

For ABA biosynthesis, cyclisation of lycopene is crucial. This reaction is carried out by two enzymes ε-cyclase and β-cyclase. The ε-cyclase adds only one ring resulting in the formation of monocyclic δ-carotene. β-cyclase adds two rings, one on each end of the linear lycopene substrate to form bicyclic β-carotene. When both of these enzymes are combined together they produce α-carotene containing one β and one ε rings on each end of the lycopene (Cunningham et al., 1996).

1.4.4 Formation of Xanthophylls

Xanthophylls are formed by hydroxylation of β or ε ring-containing carotenoids. For each ring type a specific enzyme is required for the addition of hydroxyl group to the rings (Sun et al., 1996).

Zeaxanthin is the first xanthophyll in the pathway that leads to ABA biosynthesis and it is formed from β-carotene by β-carotene hydroxylase or from violaxanthin by the action of violaxanthin de-epoxidase (VDE, reverse reaction of zeaxanthin epoxidase). The VDE requires low pH in the chloroplast lumen and is regulated to increase activity under higher light intensities. If the light levels increase rapidly a sudden accumulation of zeaxanthin is observed, this helps to avoid photooxidative damage (Rossel et al., 2002). Zeaxanthin epoxidase was the first gene to

be isolated from the ABA biosynthetic pathway (Marin et al., 1996); it catalyses the addition of two epoxy groups to zeaxanthin, forming violaxanthin. It can be regulated by light or dehydration in the roots. However, no effect on the expression of ZEP was found due to drought treatment in the leaves (Burbidge et al., 1997, Thompson et al., 2000b).

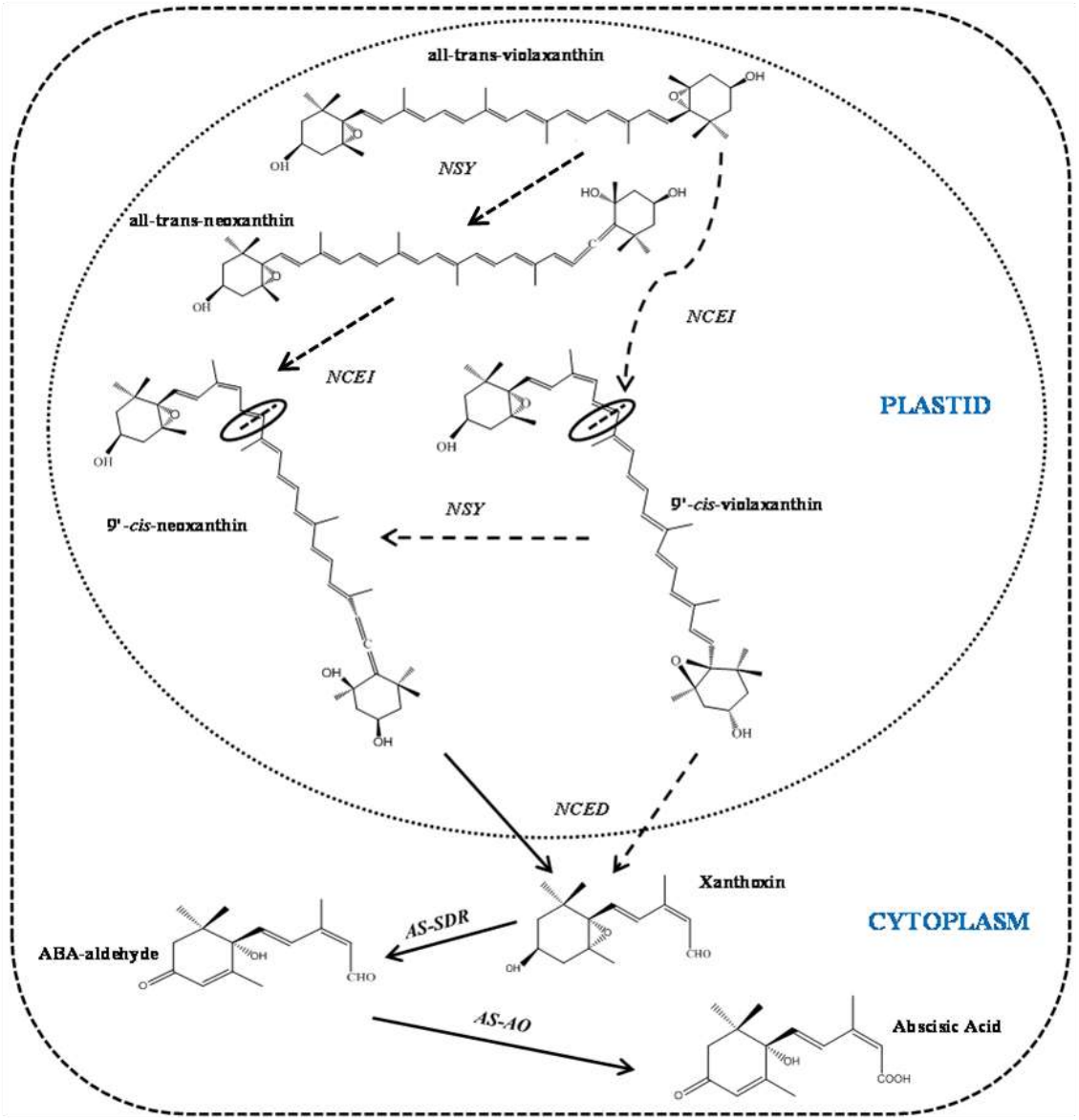

Figure 1.4. Production of ABA from all-*trans*-violaxanthin in plants. The enzyme names have been shown in bold italics; *NSY*, neoxanthin synthase; *NCEI*, 9′-*cis*-epoxycarotenoid-forming-isomerase; *NCED*, 9-*cis*-epoxycarotenoid dioxygenase; *AS-SDR*, ABA-specific short chain dehydrogenase/reductase; *AS-AO*, ABA-specific aldehyde oxidase. The dotted lines in circles indicate the cleavage position of *NCEI* on 9′-*cis*-neoxanthin and 9′-*cis*-violaxanthin. Dotted arrows indicate the steps which have not been fully characterized. From (Taylor et al., 2005).

1.4.5 Cleavage of 9-*cis*-epoxycarotenoids

9-*cis*-epoxycarotenoid dioxygenase (NCED) is a key rate-limiting enzyme involved in ABA biosynthesis. The first gene discovered to encode NCED was the maize *vp14* gene (Tan et al., 1997), a viviparous mutant that was cloned using transposon tagging. *In vitro* analysis of the recombinant protein demonstrated that it cleaved epoxycarotenoids (Schwartz et al., 1997). Later, a tomato homologue of *vp14*, now named *LeNCED1,* was cloned from a cDNA library made from wilted leaves (Burbidge et al., 1999). The *LeNCED1* was induced 8- to 18-fold as a consequence of drought treatment (Thompson et al., 2000, Tung et al., 2008, Hu et al., 2010). Further, the overexpression of genes encoding NCED has resulted in many fold increases in plant ABA contents and subsequent physiological changes (Thompson et al., 2000, Iuchi et al., 2001, Thompson et al., 2007, Zhang et al., 2008). Tomato plants transformed with *LeNCED1*, using the strong constitutive 'super promoter' (sp), showed a significant increase in ABA concentration in roots, xylem and leaves (Thompson et al., 2007) and some of these plants showed a strong phenotype with interveinal flooding and over-guttation in the leaves which was proposed to be a consequence of reduced g_s and higher root hydraulic conductivity (Thompson et al., 2007).

Subsequent studies, which over-expressed a *Phaseolus vulgaris* NCED gene (*PvNCED*) using a dexamethasone (Qin and Zeevaart) inducible promoter, showed accumulation of ABA and its catabolite phaseic acid in tobacco plants (Qin and Zeevaart, 2002). Many recent studies, for example in peanut (*Arachis hypogea* L.), have confirmed that NCED is a rate limiting enzyme and its overexpression increased the levels of ABA in plants (Wan and Li, 2006, Rodrigo et al., 2006). Further, overexpression of the monocot rice *OsNCED3* gene in the dicot *Arabidopsis thaliana*

resulted in increased ABA contents, and complementation of the *AtNCED3* null mutant, and smaller round leaves due to overexpression of *OsNCED3* was also reported (Hwang et al., 2010).

In some other studies, NCED was found to be involved in fruit ripening (Zhang et al., 2009); for example in both grapes and peach the ABA contents increased during the ripening process. It was also shown that the overexpression of *AhNCED* reduced the rate of lateral root development in *A. hypogea* (Guo et al., 2009), and that NCED overexpression increased the whole plant hydraulic conductance via aquaporins in maize (Parent et al., 2009). In tobacco it increased water and salt stress tolerance (Zhang et al., 2008) and in *Vicia faba* stomatal aperture was reduced due to *AtNCED3* overexpression (Melhorn et al., 2008).

1.4.6 Oxidation of xanthoxin to ABA

The cleavage of 9′-*cis*-neoxanthin and and 9-*cis*-violaxanthin results in the production of xanthoxin in the plastid (Figure 1.4), followed by its migration to the cytosol (Taylor et al., 2005). Then, two enzymatic reactions are involved in the conversion of *cis*-xanthoxin to ABA. The first step requires ABA specific short chain dehydrogenase/reductase (AS-SDR) (Taylor et al., 2005) which is encoded in *A.thaliana* by *AtABA2* (Gonzalez-Guzman et al., 2002). The final step in the biosynthesis of ABA is the oxidation of ABA-aldehyde to the carboxylic acid, this reaction is catalysed by ABA-specific aldehyde oxidase shown in Figure 1.4. The tomato *sitiens* (*sit*) wilty mutant is deficient in functional enzyme activity of AS-AO in the final step of ABA biosynthesis (Harrison et al., 2011). The ABA aldehyde oxidation does not appear to be a rate-limiting step in ABA biosynthesis because the AS-AO mRNA level in the leaves

was high as a result of dehydration but the activity or level of protein did not change as result of dehydration stress (Seo et al., 2000).

1.4.7 Transport of ABA in plants

1.4.7.1 ABA transport in roots

In plants, ABA can move in the symplast and apoplast (Figure 1.5). During symplastic movement the ABA anions (ABA^-) have to travel across the plasma membrane of the xylem parenchyma cells to the apoplast of the stele, and any changes in the availability of water would result in large changes in concentration of ABA in the xylem, depending on the bulk flow of water (Else et al., 1994). However, such fluctuations in xylem ABA could not be observed under field conditions, suggesting an active transport of ABA to regulate its concentration.

Several conjugated forms of ABA have been detected in the xylem sap, e.g. ABA-glucose ester (ABA-GE), where ABA is linked to glucose through an ester bond (Hansen and Dorffling, 1999), which is physiologically inactive and is stored in the vacuole; it cannot pass through the tonoplast passively, due to its strong hydrophilic nature (Sauter et al., 2002). These conjugated forms of ABA are very important as they can travel long distances within plants without any catabolic losses or enrichment in the xylem (Hansen and Dorffling, 1999). These conjugates were thought to be transported via ABC (ATP binding cassette) transporters genes (Higgins, 1992), (see detail in section 1.4.7.3).

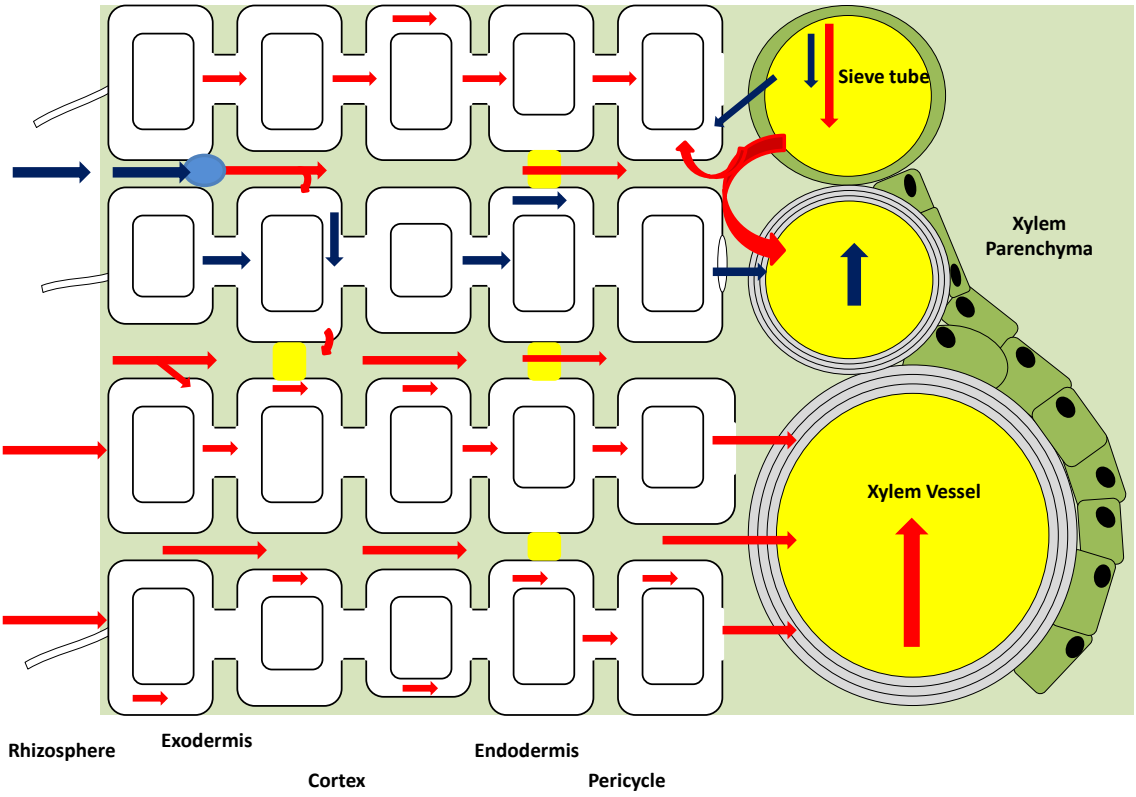

Figure 1.5. Schematic representation of ABA and ABA-GE origin and translocation in plants. Arrows indicate the direction of flow; larger arrows indicate higher flow rate. Red arrows indicate flow of ABA whereas dark blue arrows indicate the flow of ABA-GE. The blue circle indicates the enzymatic hydrolysis of ABA-GE to ABA. Yellow rectangles show the thickening of exo and endodermis (casparian band). Adapted from (Hartung et al., 2002).

1.4.7.2 Anion trap effect

ABA is a weak acid and its distribution to various compartments in a leaf depends on the pH in the apoplast (Slovik et al., 1995). ABA, like other weak acids is present in its protonated form (ABAH) when the pH is around 6.0. This form of ABA can easily pass through the plasma membrane of the cell during its journey towards the stomata due to its lipophilic nature. The protonated form of ABA can only enter the cell due to concentration gradient, this gradient in ABAH concentration is maintained by

relatively higher pH in the cytoplasm (7.2 to 7.4) which results in partial ionisation of ABA within the cytoplasm (Hartung and Slovik, 1991).

On its entrance into a more alkaline cell compartments ABAH dissociates releasing ABA^- and H^+. The dissociated anionic form of ABA (ABA^-) is lipophobic and cannot cross the membrane even if the concentration gradient exists (Kaiser and Hartung, 1981). This is hence called the ABA 'anion trap'. When photosynthesis stops at night, the pH of cellular compartments also changes as H^+ ions accumulate in the stroma; this change results in efflux of ABAH from chloroplast (Slovik et al., 1995). Through this anion trap mechanism a large quantity of ABA is removed by the leaf cells from the apoplastic sap during the active photosynthesis period as it flows along, pulled upwards by transpiration stream.

1.4.7.3 ABA transport through ABC transporters

In addition to the anion trap effect, there has been a recent discovery of ABA transporters, AtABCG25 and AtABCG40, which mediate transmembrane transport of ABA and are classified as ABC transporters. The AtABCG40 transporter is responsible for ABA uptake into the cell and is produced inside the guard cells, whereas, half length transporter, AtABCG25, is expressed in the vascular tissues of leaf. These two transporters are likely to act in a coordinated manner with AtABCG25 causing export of ABA from the site of production (Kuromori et al., 2010) and AtABCG40 importing this ABA inside the cell (Kang et al., 2010).

1.4.8. Regulation of Stomatal movement through ABA

Stomata are extremely sensitive to ABA concentration and a direct exposure of very low concentration of xylem ABA (10 to 50 nM) is enough to close the stomata (Incoll and Jewer, 1987). This suggests that some of the ABA arriving at stomata through the transpiration stream must have been filtered out as some of it is degraded in the mesophyll due to sequestration and metabolism before reaching the guard cells. Further, more ABA is stored and catabolised in the symplast from apoplast and the rate of catabolism of this ABA in the symplast is up to 5 times higher than in the apoplast (Daeter and Hartung, 1995), perhaps this was the reason that Kefu, Munns and King found nine times higher concentration of ABA in the transpiration stream than in the leaves (Kefu et al., 1991). In tomato, however, there is higher concentration of ABA in the bulk leaf than in the xylem sap as the vacuole (symplast) acts as a storage organelle. However, the correlation between leaf ABA and g_s was found to be poor (Holbrook et al., 2002). The pH gradient of the cell was thought to be one of the main driving forces of ABA diffusion as the pH increases the rate of diffusion decreases and vice versa. Similarly, as the pH of xylem increases the ability of mesophyll cells to uptake ABA reduces, hence, less ABA is sequestered/catabolised (Wilkinson and Davies, 2002). Since the discovery of ABA transporters (described in previous section) it is thought that these transporters embedded in the plasmelemma regulate the uptake of ABA, however, pH might influence the expression of AtABCG25 and AtABCG40 genes, altering the uptake of ABA.

1.4.9. ABA translocation in unstressed plants overexpressing *LeNCED1* gene

Many studies have concentrated on the mechanism of ABA action under drought conditions but little evidence (Thompson et al., 2000, Tung et al., 2008) is available where the chemical signalling mechanism of ABA has been studied under well watered conditions containing higher concentration of ABA. According to the findings described above, the protonated form of ABA (ABAH) present in the apoplast under normal pH (6.3), should not be able to dissociate to reduce the leaf g_s of plants as there are no changes in the xylem sap pH under well watered conditions. But the results from several studies on tomato (Thompson et al., 2000, Thompson et al., 2007) and in *A.thaliana* (Iuchi et al., 2001) showed reduction in the leaf g_s even under well watered conditions. Similarly, Foliar application of ABA to well watered plants reduced the g_s and increased its concentration in the xylem sap (Reviewed by; (Zhang and Davies, 1991)).

It is believed that the transgenic plants produce such a high concentration of ABA that in spite of some being catabolised or sequestered during its production and transportation, some still manages to reach the guard cells to close stomata, hence, reduce the g_s (Trejo et al., 1993).

1.5 Signal transduction of ABA

1.5.1 Linking the hormone to the target site

Though many intermediate signalling compounds have previously been characterised, the understanding about ABA signalling in plants has been limited until recently, primarily due to a lack of knowledge about ABA receptors. The discovery of PYR (Pyrabactin resistance)/RCAR, a family of soluble proteins (Park et al., 2009),

made it possible to show their interaction with ABA and PP2Cs (type 2C protein phosphatases). These proteins were found to act as 'gatekeepers' of the subclass III SnRk2 proteins, SNF1-related protein kinase-2 (Umezawa et al., 2009). The SnRK2 type gene SnRK2E was previously known to play an essential role in ABA and water stress signalling as the mutants of SnRK2E were wilty under low humidity (Yoshida et al., 2002).

The PP2Cs protein group physically interacts with SnRK2s and inactivates them by dephosphorylation of amino acid residues Ser/Thr in the absence of ABA. This step is suppressed in the presence of ABA through the RCAR/PYR receptors. Plants with inactive PP2C proteins (as in the *abi1* mutant), could not respond to RCAR/PYR receptors and inactivated SnRK2 (Umezawa et al., 2009). On the basis of results obtained from various studies a model can be drawn:

1. The ABA produced in the plant binds in a pocket of PYR/PYL proteins followed by a conformational shift so that it could isolate the hormone from the solvent phase.
2. Once bound with the hormone, the hydrophobic surfaces of the hormone are exposed on the PYR/PYL proteins.
3. On the exposed hydrophobic sites, highly conserved residues from PP2C proteins interact. If ABA is absent or cannot interact with PYR/PYL then PP2Cs are not bound. In the presence of ABA, PP2Cs are bound and PP2Cs activity is inhibited, downstream kinases (SnRK2s) remain phosphorylated, and in turn phosphorylate transcription factors activating ABA-responsive genes.

The strength of interaction between the two components of this complex depends on the sequence of PYR/PYL and any structural differences in ABA

binding pockets and on the hydrophobic PP2C interaction surface. The SnRK2s have several target sites in the cell. These include: ABI5 (ABA insensitive gene-5) which encodes basic leucine zipper (bZIP) transcription factor (Nakashima et al., 2009); ABA responsive element binding factor 2 that regulates ABA-dependent stress responsive gene expression (Lee et al., 2010), and inward rectifying K^+ channel in *A.thaliana* (KAT1).

1.5.2 Site of ABA perception

The possible site for ABA perception has been under scrutiny for the past few years, both extracellular and intracellular receptors have been suggested. The work of Hornberg and Weiler, 1984, showed that high affinity binding site of ABA in *Vicia faba* guard cell protoplasts was sensitive to trypsin (enzyme involved in protein digestion) suggesting an extracellular binding site of ABA (Hornberg and Weiler, 1984). Similarly, ABA-protein conjugates, which were unable to cross through the plasmamembrane due to their size, were able to cause altered gene expression in rice (Schultz and Quatrano, 1997). Further, in *A.thaliana* suspension cells, ion channel activity was increased due to ABA-protein conjugates (Jeannette et al., 1999). This evidence also indicates externally facing ABA receptors.

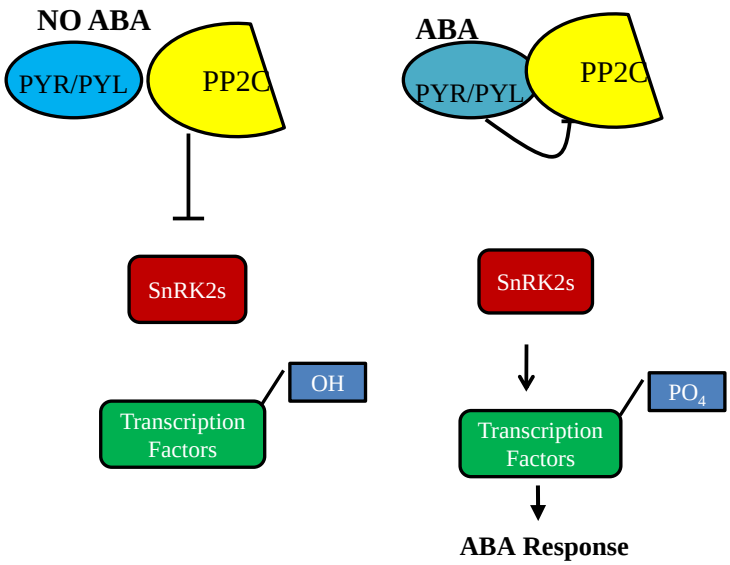

Figure 1.6. Hypothesized model of ABA signaling controlled by PYR/PYL. In the absence of ABA (left), PYR/PYL proteins are not bound to PP2Cs, so, PP2C activity is high, and so SnRK2s are inactivated by dephosphorylation, and downstream transcription factors cannot be then activated by the SnRK2s. In the presence of ABA (right), activity of PP2Cs is inhibited by binding to PYR/PYL. This results in ABA response through accumulation of phosphorylated downstream factors. Adapted from (Park et al., 2009)

On the other hand, extracellular dependence of ABA action on pH suggested as intracellular response of ABA (Anderson et al., 1994). This argument can be justified by microinjecting the guard cells with ABA which results in response i.e. stomatal closure. The recently discovered ABA receptor gene family (PYR/PYLRCAR) is found intracellularly in the cytoplasm (Park et al., 2009).

1.6 Role of ABA in various growth processes

1.6.1 Seed development and maturation

ABA has been implicated in the control of many key events during seed development and maturation. Mutant plants have helped scientists to understand the underlying genetic control of biochemical or physiological processes. For instance,

ABA deficient mutants *abi-1* and *abi-3* have revealed that the maternal ABA from the endosperm has an important role in the regulation of seed maturation in *A.thaliana* (Koornneef et al., 2002). Contrary to this, in maize (*Zea mays*), it was found that maternal ABA was not sufficient to induce seed maturation (Neill et al., 1986), but maternally-sourced ABA did enhance seed development. There was further evidence from reciprocal crosses between wild type (WT) and ABA-deficient mutants in *A.thaliana* that maternal ABA enhanced seed development (Karssen et al., 1983). ABA also helps in the prevention of precocious seed germination during mid embryogenesis (Quatrano, 1986) as demonstrated by the precocious germination that results from excision of the embryo from maternal tissue (testa and endosperm and that can be reversed by the exogenous application of ABA (Rock and Quatrano, 1995). This property of ABA is vital for crop production especially in cereals, where pre-harvest seed sprouting (i.e. precocious seed germination) can result in crop failure as seed will not be suitable for storage or consumption. ABA is also the primary source of seed dormancy; this will be described in more detail in Chapter-8.

1.6.2 Role of ABA in plant vegetative growth

The effect of ABA on plant growth is complex and contradictory. There is substantial evidence present about the inhibitory role of endogenous ABA on plant growth (Zhang and Davies, 1990), for example exogenous application of ABA resulted in reduced plant growth (Wilkinson and Davies, 2002) and high level expression of an NCED transgene led to sufficient ABA accumulation to inhibit growth (Tung et al. 2008).

However, there is evidence available about the role of ABA in promoting growth in transgenic plants over expressing NCED gene (Thompson et al 2007). Further, the mutants of ABA biosynthesis in tomato such as *flacca*, *sitiens* and *notabilis* are all stunted and near normal growth can only be achieved by exogenous application of ABA (Chen et al., 2002). There is also evidence suggesting that young rapidly developing tissues contain higher concentration of ABA than older tissues (Sweetser and Vatvars, 1976) and still grow rapidly.

The stunted growth in plants deficient in ABA is due to their inability to maintain turgor pressure as a consequence of very high water loss through transpiration (Finkelstein and Gibson, 2002) because stomata are locked open in the absence of ABA. Further, leaf epinasty (inward curling of leaves) due to excessive ethylene reduces the ability of leaves to intercept the sunlight for efficient photosynthesis, hence reduced biomass accumulation and can be considered as growth inhibitor. The ABA deficient mutants in tomato often exhibit symptoms of high ethylene genotypes, for example, plants with higher ethylene production have higher epinasty and adventitious root formation (Nagel et al., 1994) and previously, it was observed that ethylene has an inhibitory effect on plant growth (Abeles et al., 1992).

In another study on tomato mutants *sitiens* and *flacca*, it was revealed that 'normal' ABA levels were required to maintain shoot development, especially for leaf expansion in well watered plants, independent of the effect of plant water balance (Sharp et al., 2000). In the light of these findings it can be suggested that the role of endogenous ABA has two clear positive effects on growth: to antagonise the negative effect of ethylene on growth and to maintain plant water status that promotes turgor-driven growth.

1.6.3 Role of ABA in leaf expansion

Leaf area expansion is an essential part of crop productivity which can be influenced by a large number of environmental factors. There is a growing evidence about the role of water transport in plant growth (Sperry et al., 1998, Bouchabke et al., 2006), for example under root zone water stress conditions or high evaporative demand, cells cannot maintain turgor pressure, which is the major force for cell expansion (Sadok et al., 2007), however, cells can adjust their osmotic potential and hence maintain their turgor in growing tissues (Termaat et al., 1985). The uptake of water into a cell and its retention play a major role in cell growth and expansion and this is governed by the gradient of water potential in the xylem and growing cells (Tang and Boyer, 2003). Any change in the xylem water potential can directly influence the water potential of growing cells, even without any visual changes in plants. This can significantly influence leaf growth under well watered conditions.

A very high rate of transpiration can significantly reduce the rate of leaf expansion (Sadok et al., 2007), but this effect can be reversed if the plants are kept at full turgor pressure with the help of external source of pressurisation. Exogenous application of ABA or overexpression of genes encoding ABA biosynthesis enzymes can significantly reduce the rate of leaf g_s (Zhang and Davies, 1991, Thompson et al., 2000, Iuchi et al., 2001, Thompson et al., 2007) which can maintain higher Ψ_p useful in leaf expansion and elongation.

Further, it has been found that aquaporins, a large family of proteins present in the plasma membrane that act like water channels, determine the flow of water in the cell through the vacuole and between cells across the membrane (Maurel et al., 2008). The expression of plasma membrane intrinsic proteins PIP1 and PIP2 was down-

regulated in *A.thaliana* and *Nicotina tabacum* which resulted in the reduction of root hydraulic conductivity (Lp_r) of these plants, further, the ability to recover after drought stress was also impaired in these plants (Martre et al., 2002, Siefritz et al., 2002).

1.6.4 Modification of root architecture

ABA can affect the root architecture which can determine the distribution of roots in the soil (Tardieu et al., 1991). In the literature there are no examples describing the role of transgenically increased ABA levels in root architecture. However, exogenous application of ABA was reported to maintain root growth even under drought conditions by maintaining a positive turgor pressure (Saab et al., 1990). The observation that ABA reduces the total number of lateral roots in *Arachis hypogea* (groundnut) (Guo et al., 2009) helps the existing root system to grow deeper into soil for water absorption (de Dorlodot et al., 2007).

1.6.5 Increased root and leaf hydraulic conductivity

ABA is known to increase the Lp_r (Tardieu et al., 2010) perhaps by increasing the aquaporins activity (Kaldenhoff et al., 2008). Often ABA deficient mutants have reduced Lp_r (Bradford, 1983) and have poorer biomass accumulation than the wild type plants. Under high VPD the uptake of water through root system with reduced Lp_r cannot meet the transpiration demand from the canopy, this results in a reduced Ψ_p hence reduced plant growth. Contrary to this, overexpression of NCED1 in tomato increased Lp_r and was associated with increased tissue water status and leaf expansion (Thompson et al., 2007), and overexpression of NCED in maize led to a more rapid recovery of leaf elongation rate upon rewatering maize plants (Parent et al., 2009).

The role of ABA in improving leaf hydraulic conductivity through increase in aquaporins in leaves is controversial. Leaves are the major resistance to the hydraulic conductance in the above-ground parts of a plant (Sack and Holbrook, 2006), and ABA controls the aquaporins (PIP levels) in the leaves (Parent et al., 2009) increasing the hydraulic conductivity of the leaf in maize plants. However, down regulation of PIP1 and PIP2 through antisense expression did not change the hydraulic conductance of the leaves in *A.thaliana* (Martre et al., 2002).

1.6.6 Flowering

The role of ABA in the regulation of flowering was confirmed by early flowering of an ABA deficient mutant in *A.thaliana* (Martinez-Zapater et al., 1994). Flowering is induced by changes in transcript levels of flowering promoter proteins. ABA was initially thought to delay flowering by direct binding to FCA (Razem et al., 2006). This study has been controversial and the findings were subsequently withdrawn. However, ABA has been found to delay flowering through DELLA proteins activity modulation (Achard et al., 2006).

Further, it was found that the high yielding and short duration varieties of wheat had lower WUE_p due to very high g_s (Araus et al., 1993), and higher WUE_p accessions of barley, oats and wheat were found to reach flowering maturity later than with lower WUE_p (Lopezcastaneda and Richards, 1994).

1.7 Previous approaches to improve plant water use efficiency in tomato through *LeNCED1* overexpression

Much effort has been made to improve crop productivity under water-limiting conditions (Tung et al., 2008, Thompson et al., 2000, Thompson et al., 2007, Taylor et al., 2005, Tan et al., 2003, Soar et al., 2004, Huffaker et al., 1970, Blum, 1979). For example in two tomato lines (sp5 and sp12) produced at Warwick HRI by using the 'super promoter' to increase the NCED expression, the ABA level in leaves, roots, xylem sap and seeds was increased (Thompson et al., 2000, Thompson et al., 2007). High ABA levels in these sp lines had some deleterious effects, especially on seed germination, and the early phase of plant establishment (Taylor et al., 2000). These problems occurred when the "super promoter" (sp) was used to drive the transgene *LeNCED1,* (i.e. construct sp::*LeNCED1*), and might be avoided if transgene expression could be targeted only to later states of vegetative development to overcome the slow rate of seed germination. Two approaches were employed as described below.

1.7.1 Chemical genetics approach:

As described earlier in section 1.6 overexpression of NCED in tomato can result in reduced germination rates. This can be overcome by applying inhibitors of phytoene desaturase (norflurazon), but this also results in photobleaching, and then death or at least reduced establishment rates. However, by specifically blocking NCED with chemical inhibitors, endogenous ABA level in the seeds could potentially be reduced without photobleaching. The first NCED inhibitor developed was named abamine and when it was applied to cress seeds only a slight increase in radicle length was recorded (Han et al., 2004). Alternative NCED inhibitors containing hydroxamic acid groups

were developed in Dr. Andrew Thompson's and Prof. Tim Bugg's labs (Sergeant et al., 2009). The detail has been described in Chapter-7. It is proposed that in the chemical genetics approach, transgenic lines that have high ABA content, and therefore the advantages of increased WUE, but the disadvantages of slow germination, could be made more agronomically viable by applying specific NCED inhibitors to the seeds that promote germination but without interfering with subsequent growth.

1.7.2 Transgenic approach

To overcome the slow rate of seed germination observed when using the sp promoter, a promoter from *rbcS3C*, a tomato gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was used to drive NCED (Tung et al. 2008). The *rbcS3C* gene is a source of a high level of *rbcS* mRNA in photosynthetic tissues of plants under well lit conditions, but the concentration of rbcS mRNA decreases in the dark (Wanner and Gruissem, 1991).

The *rbcS3C* promoter is highly active in photosynthetic tissues (Gittins et al., 2000) and regulated by light. Its use to drive NCED did improve the rate of seed germination compared to the sp5 lines but there were signs of inter-veinal flooding and leaf yellowing, further, the leaves had a lower chlorophyll content, and plant growth was generally very slow (Tung et al., 2008). It was concluded that very high levels of ABA accumulation resulted in poor plant growth, during the early phase of plant development as with sp lines, but also later in vegetative development. The reasons for this could be manifold: direct hormonal inhibition of growth, draining of xanthophyll content by excessive cleavage by NCED, or excessive stomatal closure leading to assimilate-limited growth. Clearly more subtle approaches are needed where the dose and timing of

transgenically enhanced ABA content are optimized, and the main aim of this thesis is to use transposons to achieve random NCED expression profiles in the genome from which optimum phenotypes could be selected (detail present in section 1.8).

1.7.3 Quantitative trait loci approach to improve WUE_p

Quantitative traits are the phenotypic characteristics that exhibit a continuous distribution because the traits are controlled by the segregation of many loci, which are hence called quantitative trait loci (QTL). As many important traits are controlled by QTL (e.g., yield and drought resistance), this field of genetic research has become very important for crop improvement.

1.7.3.1 Markers assisted selection (MAS)

Molecular markers are short pieces of polymorphic DNA which are easy to locate/trace using relatively simple molecular biology based techniques; the availability of whole genome sequence in major crops such as rice, wheat, maize and tomato greatly improve the ability to identify molecular markers to map QTL associated with particular traits. For instance, for a successful marker assisted selection programme, the markers should be tightly associated with the QTL of interest and should be cost effective.

Many plant traits such as drought resistance, disease resistance and yield are complex and are controlled by multiple loci (polygenic trait); further these loci interact with the environment. The QTL studies require large population sizes and only the differences observed in the parental lines can be mapped and some loci can remain undetected due to the lack of segregation at an interesting locus (Miles, 2008).

Researchers have identified many QTL involved in the regulation of plant water use in commercial crops such as rice (Price et al., 2002, Khowaja and Price, 2008), pearl millet (Sharma et al., 2011), maize (Ribaut et al., 1996, Tuberosa et al., 2002, Szalma et al., 2007) and in tomato (Xu et al., 2008, Foolad et al., 2003). In *A.thaliana* a QTL which controls WUE was proposed to be caused by the underlying ERECTA gene (Masle et al., 2005), a putative leucine-rich receptor like kinsae (Torii et al., 1996).

In contrast, the transgenic approach is robust and directed, but knowledge of genes conferring useful functions is required in advance. A trait of interest can be selected and targeted in comparatively short period of time. The plants can be transformed with the gene of interest and its effects on plant phenotype and genotype can be compared in two to three generations. For complex traits, and for genes with pleiotropic effects, transgene expression levels and patterns are critical to obtain the desired phenotypes. Thus during the present study transposable elements carrying the transgene of interest were used to novel generate variation in transgene expression with the aim of optimising WUE and plant growth. The characteristics and uses of transposable elements are therefore described in the next section.

1.8 Transposable elements

Transposable elements (TEs) are found to be ubiquitous in most living organisms. In some organisms such as maize, it is estimated that more than 50% of the genome constitutes TEs (SanMiguel et al., 1996). The maize *Ac* and *Ds* elements were first identified by Barbara McClintock during 1947 while working on the mechanism of inheritance of mosaic colour pattern in maize kernels. In her work she showed that the mosaic pattern in maize was due to transposition of DNA segments within the genome

which she referred as 'controlling elements'. The movement of an activated *Ds* element into an exon of the anthocyanin controlling gene resulted in the disruption of gene function hence colourless aleurone layers were present in the maize kernels.

TEs have been classified into families, and on the basis of their structure and mode of transposition. TEs have also been grouped into two distinct classes (Finnegan, 1992) described below;

1. Class-I transposons: These TEs need reverse transcription in order to copy the RNA into DNA. The class-I TEs can be further divided into retrotransposons, which can be identified by their long terminal inverted repeats (LTRs), and short and long interspersed elements (SINEs and LINEs, respectively).
2. Class-II transposons: these are also known as DNA transposons, these TEs are mobile and use single stranded or double stranded DNA (Craig, 2002). DNA transposons can be sub divided into three categories: (a) *Ac/Ds* transposons which have the ability to excise as double stranded DNA and reinsert elsewhere in the genome and will be described further in the forthcoming sections as *hAT* super family; (b) rolling circle replication transposons, known as helitrons (Kapitonov and Jurka, 2001) and (c) mavericks which contain integrase-like proteins which help in the integration of double-stranded DNA transposition intermediates (Pritham et al., 2007).

1.8.1 The *Ac* element from maize

Interest in the maize transposable elements increased during the last quarter of the 20th century, primarily due to availability of genome sequence data. The sequences of the first two *Ac* elements isolated from insertions within the waxy gene of maize

(Fedoroff et al., 1983, Behrens et al., 1984) revealed similarity to each other (Pohlman et al., 1984).

The *Ac* element of maize belongs to *hAT* super family of DNA transposons. The term *hAT* has been derived from; *h*, hobo from *Drosophilla melongaster*; *A*, *Ac* element from maize and *T*, Tam3 derived from *Antirrhinum majus* (Calvi et al., 1991). All members of this superfamily encode transposase *(Tpase)* genes which possess 20-60% or even higher protein sequence homology. Some of the other members included in this family are Ascot-1 (from the fungus *Ascobulus immersus*), hermes (present in the house fly *Musca domestica*) and hopper (discovered in the oriental fruit fly, *Bactrocera dorsalis*).

Members of *hAT* super family show a highly conserved amino acid sequence for the encoded *Tpase* (Rubin et al., 2001) and only contain one *Tpase* gene, unlike other class-II transposons encoding more than one protein to catalyse and regulate the transposition (e.g., Tn7 and Mutator transposons) (Gray, 2000).

1.8.1.1 Sequence characterisation of *Ac* element

The maize *Ac* element comprises of three open reading frames (ORF) which occupy most of the 4563 bp length of this element: ORF1 and ORF2 overlap such that the start codons (AUG) are 431bp apart and are in divergent orientation to ORF3. The ORF1 is 807 amino acids and encodes the *Tpase*. The N-terminus of *Tpase* at residue 102, contains one of the three nuclear localisation signals in the protein (Boehm et al., 1995). This terminus also contains a DNA binding domain. If this region is deleted, the import of *Tpase* from the nucleus is greatly reduced, hence this region is vital for transposition (Li and Starlinger, 1990). The C-terminus contains highly conserved

amino acids, including a dimerisation domain and possibly catalytic residues. The C-terminal sub-domain is sufficient to bind the repetitive sequence motif present in the element sub termini (Becker and Kunze, 1997).

The *Ac* element contains sequences that act both in *cis* and *trans* to promote transposition. The *trans* acting regions starts at around 300 bp from the 5′ end and terminates at approximately 264 nucleotides from the 3′ end (Kunze et al., 1987).

It was astonishing to find that there was very little affinity between the TIRs and *Tpase*, though *Tpase* always binds to the TIRs. The TIRs have no similarity to the sub-terminal binding sites. However, both N and C-termini sub domains are required for recognition and the transposition to occur. Any changes due to substitution, insertion or deletion will result in the loss of *Tpase* function.

1.8.1.2 Interaction of *Tpase* with *Ds* element

Barbara McClintock found that on a particular site on a short arm of maize chromosome 9 there was frequent breakage. This was because of a result of dissociation. The term dissociation refers to the ability of the *Ds* element to transpose (jump) in the presence of the *Ac* element. However, the *Ds* element was unable to dissociate in the absence of *Tpase* provided by the *Ac* element. The *Ds* element originated from internal deletion in the *Tpase*, which rendered it unable to transpose independently (Baker et al., 1986). As the *Ds* element lacks functional *Tpase*, its ability to transpose can be restored in the presence of an *Ac* element. In *trans*, this can only be achieved if the *Ds* element still retains its TIRs. Apart from the 11-bp TIRs, there is often little homology between *Ac* and *Ds* elements (Sutton et al., 1984). The 11-bp TIRs and the sub-terminal regions

approximately 250 bp from 5′ and 3′ ends are required in *cis* for transposition (Varagona and Wessler, 1990) in the *Ac* and *Ds* elements.

As previously mentioned, the *Tpase* binds to 11-bp TIRs present in the *Ds* element and a few very short sub terminal repeats (Coupland et al., 1988, Varagona and Wessler, 1990). This process is very specific because any substitution in the TIRs prevent *Ac* recognition of the *Ds* element (Becker and Kunze, 1997).

The presence of only one *Ac* element, encoding the *Tpase*, is necessary for excision of the *Ds* element (Coupland et al., 1988). In maize, as the *Ac* copy number increases, the frequency of transposition decreases (McClintock, 1950). This reduced *Ds* excision frequency might be due to methylation of *Ac* transcripts (Chomet et al., 1987). However in tobacco, the opposite is true (Hehl and Baker, 1989), but in *A.thaliana* it was found that the transposition frequency of the *Ds* element was directly proportional to the level of mRNA provided by *Tpase* (Swinburne et al., 1992). This suggests that the quantitative effect of the *Ac* element on *Ds* transposition might be host specific.

1.8.2 Characterisation of the *Ds* element

As previously described in section 1.8.1.2, the *Ds* element is functionally defective due to lack of 0.2 kb in the coding region of *Tpase* and it can arise directly from deletion mutations in an *Ac* element which suggests that both *Ac* and *Ds* elements of maize were structurally related (Fedoroff et al., 1983, McClintock, 1955). The *Ds* element (2.0 kb in size) found in the maize *waxy* locus had homologous sequence at both of its termini. Further, its endonuclease cleavage map was also found to be identical to the *Ds* element from the *shrunken* locus, (Courage tebbe et al., 1983), which

suggested that the *Ds* element was mobile in the presence of an *Ac* element providing the functional *Tpase*.

At least 30 DNA sequences from maize were found to have homology to the *Ds* element (Sutton et al., 1984). The two *Ds* elements, *Ds1* and *Ds2* can affect the plant phenotype depending on the host genome. The names of these two elements coincidently match with the TIR sequence of *Ds1* and *Ds2* elements used in the present study which are a part of a single *Ds* element. The *Ds1* element cloned from maize locus *alcohol dehydrogenase* (*adh1*) was described by (Sutton et al., 1984), insertion of *Ds1* element in the *adh1* locus might be responsible for the reduction in *adh1* transcripts (Dennis et al., 1988). This might be due to the presence of extra sequence from *Ds1* element which acts like an intron or inefficient splicing of external intron. However, the insertion of *Ds1* element did not disrupt the code for normal polypeptides (Dennis et al., 1988).

On the other hand, type-2 *Ds* element (Merckelbach et al.) cloned from maize shrunken locus (Giroux et al.) described by (Giroux et al., 1994), encodes as subunit of starch (ADP-glucose pyrophosphorylase), can splice out by itself, without *Tpase*. In this case, the *Ds2* element uses the splice signal from its host genome (Dennis et al., 1988).

1.8.3 DNA transposons: A source of allelic diversity

In the genomic evolution of species, generation of allelic diversity and creation of novel regulatory sequences are the major underlying forces and this allelic diversity can be caused rapidly by TEs. Insertion of a transposed *Ds* element can affect the host genome in many ways (Kidwell and Lisch, 1997, Wessler, 1988). For example, insertion of the *Ds* element can disrupt gene function through transposition into the coding

sequence of a gene. However, transposition into a promoter or intron may alter the phenotype of the plant due to the change in the gene regulation.

The ability of *Ac* and *Ds* elements to transpose near to, or within, a gene has been exploited by geneticists to tag the genes, which has been a widely used system to find a particular gene of interest associated with the phenotype of the plant (Cooley and Yoder, 1998, Altmann et al., 1995, Kidwell and Lisch, 1997). The DNA transposons such as *Ac/Ds* elements can undergo spontaneous excision in the presence of *Tpase* resulting in the reversal of the phenotype, so the TEs can generate reversible and unstable mutations in the host genome. If the source of *Tpase* is removed by out-crossing with a wild type plant the transposition of the *Ds* element can be prevented, and the phenotype is stabilised in some of the resultant progeny.

1.8.4 TEs as a source of large scale chromosomal restructuring

TEs can generally undergo simple transposition events, where a small part of the DNA is transposed, but the observation that kernels showing the phenotypic variation were also unable to germinate, suggested that the TEs can result in lethal rearrangements of the chromosomes (McClintock, 1950).

In the intra-chromosomal rearrangements whole segments of a chromosome can be duplicated between the donor and recipient site, which might be present in direct or indirect orientation (Figure 1.6). It has been reported that chromosomal inversions, duplications and deletions of up to 100 kb are possible in TE rearrangements (Gray, 2000, Lim and Simmons, 1994, Zhang and Peterson, 2004).

1.8.5 Transposition of macrotransposons

Transposon pairs in direct orientation can generate numerous heritable chromosomal rearrangements. One such rearrangement is the transposition of 'macrotransposons' which are a large DNA segment (up to 100 kb) and extend from the 5′ end of one chromosome to the 3′ end of another chromosome (Huang and Dooner, 2008). Macrotransposons transpose when two separate *Ds* elements are recognised by the same *Tpase*.

Similarly, through insertion of a *Ds* element in another *Ds* element, the chromosome involved breaks at this point due to formation of a dicentric chromosome (Kunze and Weil, 2002). The presence of pairs of TEs in close proximity can result in chromosomal breakage even if they are more than 100 kb apart (Dooner and Belachew, 1991) and are able to recognise TEs up to 25 times bigger than their own length (Huang and Dooner, 2008). Such a big chunk of DNA on splicing from one site and its reinsertion into another can generate deletions, inversions and reshuffling of the host genome. The macotransposons can carry some intergenic regions (Ralston et al., 1989), which suggests that transposition of a macrotransposon might also carry several genes present between the two TEs. This can eventually result in changes in gene expression or the birth of new genes.

1.8.6 Epigenetic silencing of new transposons

In order to avoid damaging mutagenesis that might be caused by transposons, the host genome has evolved mechanisms to silence these active transposons. Further, for transposons to persist, it is vital that the *Tpase* remains active. The balance is often reached by epigenetic silencing of TEs, which is usually reversible. Recently, many

genes involved in epigenetic control of active transposons have been characterised, including genes encoding DNA methyl transferases and demethylases, chromatin remodelling enzymes, and genes involved in small RNA metabolism (Dooner and Weill, 2007, Henderson and Jacobsen, 2007). Despite these transposon silencing mechanisms in plants, some transposons have still managed to increase their copy number in plants before becoming silent again (Vagin et al., 2004).

1.8.7 Upregulation of transposon activity

Transposon activity has been reported to increase under stress conditions, for example, after wounding in *Nicotiana tabacum* (Mhiri et al., 1997); osmotic stress with sucrose (Iantcheva et al., 2009) or salt in tomato (Tapia et al., 2005), reactive oxygen species in *Medicago truncatula* (Stoycheva et al., 2010), and gamma radiation in *Saccharomyces cerevisiae* (Sacerdot et al., 2005). It can be hypothesized that under stress conditions, transposons are transcriptionally activated to help in adaptation of the host in changing environmental conditions.

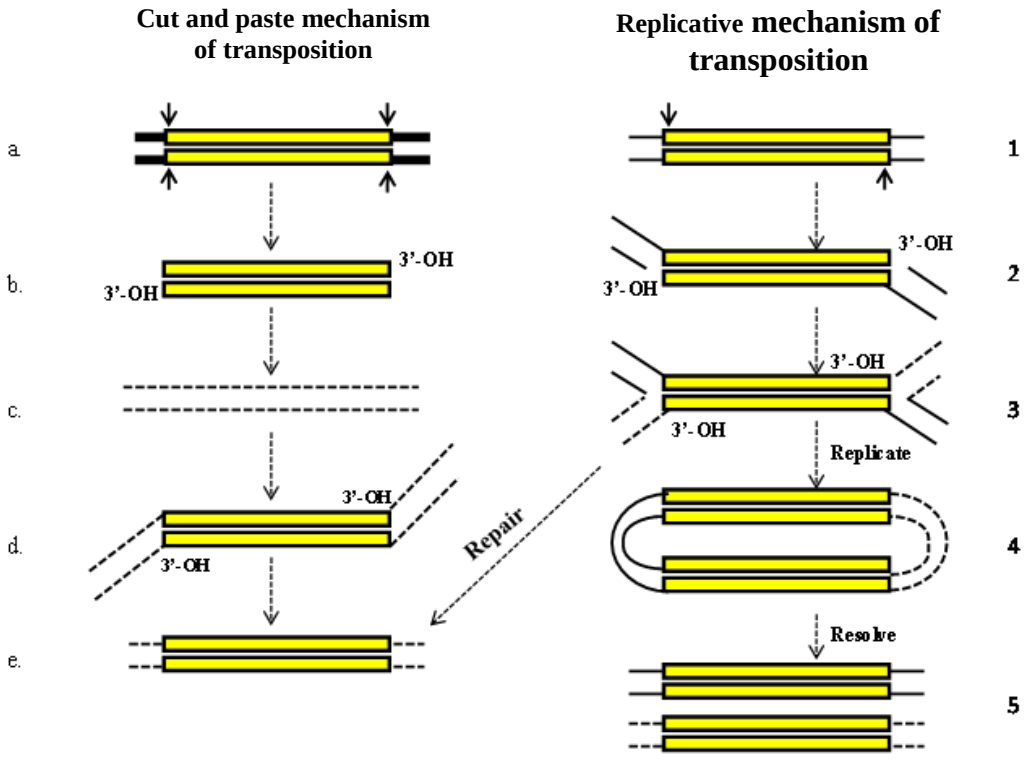

Figure 1.7. Mechanism of transposition in transposable elements. Solid filled lines shows host DNA; dashed lines, recipient DNA; yellow rectangles, *Ds* element; solid arrows, cleavage points by transposase. Left, cut and paste mechanism of transposition: a, binding of transposase to the *Ds* element; b, cleavage of transposase generates 3′-OH group on each end of the *Ds* element; c, binding of transposase to the target sequence; d, transposase uses 3′-OH group to invade for strand transference reaction as a result 8-bp target site duplication occurs; e, completion of insertion of *Ds* element to the new location. Right, 'replicative mechanism of transposition': 1, only one strand of DNA is nicked by transposase; 2, generation of 3′-OH group cleaves and make nicks on target DNA; 3. Binding of transposase to the target sequence, note that donor DNA has not been released completely and one strand is still covalently bound to the donor site; 4, donor DNA stays intact and duplication of newly inserted DNA occurs so that one end is attached to donor DNA and the other attached to target DNA; 5, enzymatic resolution of these elements occurs resulting in two copies of *Ds* element. Adapted from (Mahillon and Chandler, 1998).

1.8.8 *Ac/Ds* transposition as a tool to generate large populations of transgenic plants with variable expression of a transgene

In the *Ac/Ds* transposition system, the *Tpase* ORF is substituted with a desired gene of interest to create a *Ds* element containing a gene of interest with the desired regulator elements at the 5′ and 3′ ends. The other component is an *Ac* element carrying a functional *Tpase* ORF with truncated TIRs so that it is fixed (non-mobile) in the genome but has the ability to induce transposition in *trans.* These modified *sAc* and *Ds* elements can be introduced in the plant genome through *Agrobacterium*-mediated transformation. The modified *Ds* element can be activated by cross pollination of plants containing the *sAc* element, which provides *Tpase* to reposition/transpose in the genome.

The maize *Ac/Ds* element-mediated repositioning of a transgene can be a very useful technique to produce a large population of plants, each with varying levels of transgene expression. The approach also allows the transgene to be separated from linked selectable markers (usually antibiotic resistance genes) and other T-DNA components, in compliance with the EU transgenic crops directive 2001/18/EC.

Another key advantage of using the *Ac/Ds* element system might be the ability of transposons to give higher and more stable gene expression as found in *Solanum tuberosum* nuclei (Yan and Rommens, 2007), compared with the plants transformed with a T-DNA backbone. This might be due to a higher number of T-DNA insertions in the protoplast as compared to individual cells. This observation suggests that the *Ds* element integration preference is towards genomic regions that promote higher level of expression.

The transposition of *Ds* elements is unpredictable but the high excision frequency of this element and its successful re-integration in the *Solanum lycopersicum* genome (Burbidge et al., 1995) and the ability of *Ds* element to transpose near to enhancer or within transcriptionally active regions means that a large population of transgenic plants overexpressing the gene of interest can be produced with variable expression levels by using only one cross between *Ac* and *Ds* element-carrying plants. This helps to avoid repeated transformations, laborious and time consuming tissue culture with potentially low recovery of the transgenic plants, and saves time, manpower and precious resources.

The first report of using the *Ac/Ds* elements as a vehicle to introduce genes into a heterologous system was the introduction of a 247-bp fragment of the maize waxy gene using *Ac* element in tobacco protoplast. This resulted in nearly 50% of the *Ac* copies carrying the gene transposing in the genome (Baker et al., 1986).

To avoid the autonomous transposition of the *Ac* element, TIRs of this element can be truncated. This *Ac* element is called 'stabilised activator' (*sAc*). In a study conducted in tomato by Carroll et al. (1995), *sAc* was cross-pollinated with the plants containing *Ds* element. This resulted in F_1 progeny containing both *sAc* and *Ds* element in the same genome which caused the transposition of *Ds* element to different positions in the genome (Carroll et al., 1995). Since then many researchers have made use of this principle to transpose a non-autonomous *Ds* element carrying genes of interest (Goldsbrough et al., 1993). Transposon-mediated generation of T-DNA- and marker-free rice plants expressing a Bt endotoxin gene (Lisson et al., 2010).

In this thesis, such a system was designed in which tomato (*Solanum lycopersicum* L.) plants were transformed with *sAc* and *Ds* element. The non-functional

Ds element's ORF was replaced by the *LeNCED1* gene controlled by the Histone H2A promoter. The aim was to generate variation in expression in NCED such that increases in WUE could be obtained whilst avoiding transgene expression at times or to levels that would reduce yield potential.

1.9 Aims and objectives

There were two main aims:

1. To generate novel variation in the expression of NCED by transposition of a Histone H2A::*LeNCED1* transgene, allowing NCED to randomly integrate into new positions in the tomato genome and then screening of transgenic plants to select genotypes with improved WUE and productivity.
2. To evaluate the possible utility of novel NCED inhibitors for improving seed germination and seedling growth rates of transgenic lines with enhanced ABA biosynthesis.

The first aim was achieved by addressing the objectives below:

- Generation of F_2 seed from the cross between the stabilized Activator (*sAc*) and *Ds* element (Ds1::histoneH2A::*LeNCED1*::Ds2).
- Selection F_2 plants that retain the *Ds* element, but that have lost the *sAc* element, and so have stable and heritable integration of the *Ds* element in plant genome.
- Selecting plants lines with normal seed germination and plant establishment, but low stomatal conductance and improved long-term WUE.
- Determining the copy number and inheritance pattern of *Ds* elements in selected lines using Southern blotting and quantitative PCR.

- Determining the level and pattern of NCED expression in selected line/s that is responsible for favourable phenotypes.

The second aim was accomplished by the following objectives:

- Evaluating the effect of different concentrations of hydroxamic acid NCED inhibitors on rate of seed germination and seedling vigour on various difficult-to-germinate tomato genotypes.
- Using these chemicals to study the role of ABA in post-germination plant establishment, growth and development in tomato.

CHAPTER-2

MATERIALS AND METHODS

2.1 Plant material used during the experiment

2.1.1 Seed germination assay

Tomato seeds were surface sterilised using 10 percent household bleach (Domestos) for 30 minutes and washed three times in sterilised water. Petri dishes of 90 mm diameter and 20 mm depth were used to germinate the seeds. Sterilised Whatman No.1 filter papers were used as a bedding surface for the seeds. To maintain the optimum moisture for seed germination, 1.5 ml sterile deionized water (dH_2O) was used in each Petri dish to soak the filter paper. The Petri dishes were sealed with paraffin film to maintain the humidity and were kept in the dark at 25^{o}C until the emergence of radicles. For high ABA seeds such as sp5 (sp5:*LeNCED1*), seeds were incubated overnight on Whatman No.1 filter paper soaked in appropriate concentration of norflurazon to improve the rate of seed germination. Seeds were washed with sterile dH_2O three times and again incubated in the dark on filter paper wetted with 1.5 ml sterile dH_2O. For F_2 (self pollination of *sAc* × *Ds* line) seeds, D4 Hydroxamic acid (1.0 mM) (Sergeant et al., 2009) was used to obtain a uniform seed germination.

2.1.2 Sowing in compost

Upon germination seeds were sown in F2s compost (Levington Horticulture, Suffolk, UK) in FP-24 trays, once germinated seedlings were transferred to 10-cm square pots containing M2 compost (Levington Horticulture, Suffolk, UK). Three-week

old plants were then transferred to into 20-cm pots in M2 compost and approximately 6-weeks old plants were transferred into 25-cm pots containing M2 compost.

2.1.3 Control plants

During the experiment, control plants i.e. sp5 and tomato mosaic virus resistant line of *Solanum lycopersicon* L., previously known as *Lycopersicon esculentum* M. 'Ailsa Craig Tm2^{a}' was used as wild type (WT) control. The second group of control plants constituted of sp5 plants, this true breeding genotype was included in the experiments because the plants from this genotype were known to have reduced stomatal conductance (Thompson et al., 2007).

2.1.4 Tomato Seed extraction

Tomato fruits were harvested at breaker stage (Orange/yellow or some sign of red colour) and cut diagonally into two pieces for convenient seed extraction. The cut fruits were squeezed to remove the seeds into a clean labelled container. The extracted seeds also contained the flesh, jelly and tomato juice; these were digested using Pectinase (Young's Brew Pectolase, Bradley, UK, Ltd). Acid Pectinase helped degradation of tomato fruit flesh and jelly around the tomato seeds. The extracted seeds were left in the acid pectinase solution overnight. Seeds were transferred to labeled hand held sieves and separated from the digested jelly by washing under running cold water. Seeds were left to dry in the lab for at least one week to avoid any microbial growth and germination during storage due to excessive water contents. Dried seeds were then packed in the labeled envelopes and stored at 4^{o}C, 25% relative humidity. Acid pectinase was prepared in 0.25 M Hydrochloric acid 1.0 mg ml^{-1} pectinase.

2.1.5 Nutrient film technique for seedling growth

This method was only used for the germination of F_2 (selfing of *sAc* × *Ds*) plants. Soon after the emergence of the radicle, seeds were transferred on to Rockwool blocks. A small hole was made in each Rockwool block and germinated seeds were carefully transferred to avoid damage to the radicle. Each Rockwool block was then covered using vermiculite and thoroughly irrigated with nutrient solution. The nutrient medium contained two types of the stock solutions, A and B detailed below.

Stock Solution-A: To make up the solution to 100 litre;			
Chemical	**Quantity**	**Molecular Weight**	**Concentration (M)**
Potassium nitrate (KNO_3)	400 g	101.1	0.064
Magnesium sulphate ($MgSO_4.7H_2O$)	570 g	246.47	0.023
Mono potassium phosphate (KH_2PO_4)	250 g	136.08	0.018
Zinc sulphate ($ZnSO_4.7H_2O$)	225 g	287.53	0.008
Manganese sulphate ($MnSO_4.H_2O$) 32%	3.0 g	169.02	0.00012
Boron (Borax) $Na_2B_4O_7 \cdot 10H_2O$ (11%)	2.25 g	381.37	0.00005
Copper sulphate	0.3 g	249.68	0.000012
Ammonium molybdate ($(NH_4)_6Mo_7O_{24}$)	0.075 g	1235.86	6.1×10^{-7}

Stock Solution-B: To make up to 100 litre volume;

Chemical	**Quantity**	**Molecular Weight**	**Concentration (M)**
Calcium Nitrate $Ca(NO_3)_2.4H_2O$	400 g	164.08	0.017
Calcium chloride ($CaCl_2.2H_2O$)	325 g	147	0.027
Potassium nitrate (KNO_3)	650g	101.1	0.064
Iron diethylenetriaminepentaacetate (DTPA) 7%	75g	468.2	0.0017

In order to prepare 100 litre of the nutrient solution, 1000 ml of stock solution-A was dissolved in about 10 litres of water, followed by addition of 1000 ml of stock

solution-B diluted to the required volume. Two stock solutions were prepared and diluted separately to avoid precipitation.

It was observed that if the solution was continuously exposed to sunlight there was a rapid algal growth which could cause poor plant growth in the nutrient medium. To avoid this, an opaque plastic film was used to cover the nutrient storage tank from the direct exposure to the sunlight.

2.1.6 Plant propagation through cuttings

Cuttings were obtained from side shoots approximately 10-15cm in length. These shoots were again recut with the help of a sharp knife under water and potted in 10-cm pots so that one third of the cutting was in the medium. These pots contained M2 compost (85%) and vermiculite (15%) for better soil aeration. The pots containing the cuttings were sealed with plastic bags with few holes to maintain a high humidity environment around the cuttings. After about 3-4 days, these bags were slit from one side and approximately two weeks old cuttings were transplanted in 20-cm pots containing M2 compost.

2.1.7 Cross pollination

Tomato flowers were mainly emasculated preanthesis at semi open flower bud stage. Pollens were collected in a small glass vial from fully open flowers with the help of an electric buzzer. After about two days of flower emasculation, freshly collected pollens were applied on stigma with the help of a paintbrush. This process was repeated to achieve a successful fertilization.

2.1.8 Plant growth and environmental conditions in the glasshouse

The day temperature was set at 22^{o}C and night temperature at 18^{o}C. During the winter season (October to April), plants were grown under supplementary lighting for 16 hours a day (0200 hrs to 1800 hrs). These hours were chosen to reduce the carbon foot print. If the plants were to be taken to the Weiss room to collect their stomatal conductance data, the lighting period in the glasshouse was adjusted one week prior to their transference to the Weiss room to match the circadian rhythm of the plants with the Weiss room daylight hours (0700 hrs to 2100hrs). Plants were irrigated by hand to well watered conditions throughout their growth period. Occasionally plants were sprayed for mildew and blight control by Horticultural Services staff.

2.1.9 Controlled Environment (Weiss room) Conditions

Weiss room specifications:

Plant Growth Area:	300 x 300 cm
Maximum head Height:	300 cm
Lamp Type:	HQI (Hydrargyrum Quartz Iodide)

Following conditions were set during the course of the study in the Weiss room

Vapour Pressure Deficit (VPD) day	0.92 kPa
Vapour Pressure Deficit (VPD) night	0.72 kPa
Temperature day:	22^{o}C
Temperature night:	18^{o}C
Relative Humidity Range:	65%
Irradiation:	400 μmol m^{-2} s^{-1}
CO_2 concentration:	360 ppm
Photoperiod:	16 hrs (0700 hrs to 2100 hrs)

2.2 Gravimetric water use measurements

To quantify the total water losses through transpiration in different genotypes, the exposed surface of pots containing these plants were wrapped with the help of Clingfilm 11μm thick. A blank pot (without a plant) was used in each block, instead of a plant a stick of approximately similar diameter as of the plant's stem was used to mimic similar pots containing the plants. These plants were weighed every morning and watered to the field capacity, if required. Water loss from the blank pots was deducted from each pot at the end of experiment, this water loss determined evaporative losses through the cling film. Previous gravimetric experiments conducted in Dr. Andrew Thompson's lab showed that wrapping of pots with the cling film did not affect the plant growth.

2.3 ABA quantification

2.3.1 Sap collection

Xylem sap collected from tomato plants at different growth stages. In two week old plants, the stem was cut with the help of a sharp scalpel blade about 3.0 cm from soil level. Silicon tubes (5 cm in length) were fitted snuggly onto the stumps. These pots were placed in pressure chambers. The sap was forced out of the cut stem by increasing the air pressure in the chamber, and collected in 1.5 ml amber micro centrifuge tubes. These tubes were placed in polystyrene box containing liquid nitrogen and covered with aluminum foil to avoid ABA degradation due to light.

In case of bigger tomato plants (4-8 weeks old) stems were cut with the help of sharp knife leaving approximately 5.0 cm stump followed by sealing with silicon tubing. Sap was collected with the help of pipette in a screw cap 2.0 ml tubes. These tubes were

immediately stored in liquid nitrogen in the dark. During sap collection the exudates appearing in the first instance was discarded.

2.3.2 ABA extraction from leaf Samples

The terminal leaflets from first fully expanded leaves from each plant were harvested in the morning half an hour after irrigation. These leaflets were wrapped in aluminum foil and stored immediately in liquid nitrogen followed by storage at -80^{o}C, until processed.

2.3.2.1 Bulk leaf Extract for ABA quantification

Exactly 10 mg freeze dried tissue was weighed in 2 ml amber micro centrifuge tubes. A tungsten carbide bead (3 mm in diameter) was added in each tube. This was followed by addition of 400 µl of solvent-A and 5 µl of internal standard. The tissue in the tubes was disrupted by using a bead beater (Qiagen, UK, Ltd) for 2 minutes at 25 Hz s^{-1}. These tubes were centrifuged at 13000 rpm for 10 minutes. The supernatants were transferred in to a new amber micro centrifuge tube and placed on ice in the dark, leaving the pellet undisturbed.

In the pellet obtained during the previous step, 400 µl of solvent-B was added followed by homogenization for 2 minutes at 25 Hz s^{-1}. The homogenate was centrifuged for 10 minutes at 13000 rpm. The supernatants obtained were mixed in the supernatants obtained in the last step. The contents were thoroughly mixed and centrifuged for 1 minute to avoid any tissue debris being carried through to the next step. This material was transferred to an auto sample vial and stored in -80^{o}C freezer

until final processing. For every 24 samples one sample was prepared containing all other reagents except bulk leaf sap, this was done for standardization purpose.

Sap samples were thawed out on ice followed by centrifugation at 13000 rpm for 2 minutes. These samples were transferred in to a new auto sample vial and stored in -80^{o}C freezer until final processing.

All processing was carried out under minimum light conditions and every effort was made not to expose these samples for too long to the light. These samples were processed by Mark Bennett at Imperial College London by using LC/MS/MS (Forcat et al., 2008).

Solvent-A

10% Methyl Alcohol (HPLC grade)

87.75% dH_2O (MilliQ grade)

1% Acetic acid (Analar)

1.25% internal standard (1 ng $^{2}H_6$ ABA)

Solvent-B

10% Methyl Alcohol (HPLC grade)

89% dH_2O (MilliQ grade)

1% Acetic acid (Analar)

2.4 Histochemical staining for GUS activity

Histochemical staining reactions were performed in GUS staining buffer. The buffer was eluted in 96 well plate (200 µl per well), root samples approximately 0.5-1.0 cm in length were excised from the F_2 plants growing in the nutrient film. These

samples were washed in dH_2O followed by immersion in to the GUS staining buffer. These plates were sealed with aluminum foil to avoid solution evaporation. Samples were incubated at 37^oC overnight and visualized for GUS activity. The plants found to be GUS negative were again screened for GUS activity before transplantation in to the soil medium to avoid any false negatives.

2.4.1 GUS staining buffer

Gus staining buffer contained 1.0 mM of 5-Bromo-5-Chloro-3-Indolyl-β-D-glucoronide, 50 mM Sodium phosphate buffer pH 7.0, 4 mM potassium (+) Ferrcynide, 100 $\mu g\ ml^{-1}$ chloromphenicol and 0.1% triton X-100.

2.5 Plant water relations

2.5.1 Leaf Water potential

Measurement of water potential (Ψ_l) of well watered plants was carried out in the morning approximately 2 hours after the sunrise. A terminal part of the leaf containing three leaflets from first fully expanded leaf was harvested and sealed in the plastic bag (Wenkert et al., 1978, Turner and Long, 1980). Each leaflet was cut with the help of a sharp scalpel blade and Ψ_l was measured by using the pressure chamber so that the cut end of the petiole was protruding from the chamber and exposed to the atmospheric pressure. The pressure in the chamber was increased very slowly, until a liquid column could be seen at the severed end of the petiole (Millar and Hansen, 1975).

2.5.2 Osmotic potential

Plant osmotic potential (Ψ_s) was measured of the same leaves which were used for the measurement of Ψ_l. These leaves were wrapped in aluminum foil and stored in liquid nitrogen and frozen at -80^{o}C until being processed. Frozen leaflets were inserted in to a 20-ml syringe and allowed to thaw. A plunger was inserted in to the syringe and the extracts were forced out and collected in 2.0 ml micro centrifuge tube. The extract was centrifuged briefly to get rid of debris and measurements were made by using an osmometer (Cam Lab UK Ltd.); 100 µl of extract was used for each measurement and at least three measurements were made for each sample and averaged across the samples. The readings obtained were converted from mosmol kg^{-1} H_2O to MPa by using the following formula (Ben Hassine et al., 2008) ;

$$\Psi_s \text{ (MPa)} = - C \text{ (mosmol kg}^{-1} H_2O) \times 2.58 \times 10^{-3}$$

In this equation 'C' is the osmolarity of the tissue in mosmol kg^{-1} H_2O.

2.5.3 Leaf turgor pressure

Leaf turgor pressure was determined by using the following formula

$$\Psi_p \text{ (MPa)} = \Psi_l \text{ (MPa)} - \Psi_s \text{ (MPa)}$$

2.6 Plant growth measurements

Petiole length (cm) was measured by using a meter ruler; the leaves were detached from the stem before the measurements. Leaf area (cm^2) was measured by using leaf area meter (Delta T device, MK2, Cambridge, UK). Plant dry matter contents were measured by incubation of tissues at 80^{o}C for 72 hours, all above-ground tissues (leaves, stem, petioles and inflorescence) were packed separately in paper bags prior to

incubation and this was followed by reweighing the tissues to get the dry weight of the tissues. The net assimilation rate (g $cm^2 day^{-1}$) was calculated by the following equation (Hunt et al., 2002);

$$\text{NAR} = \frac{(w2 - w1)}{(LA2 - LA1)} \times \frac{(\log LA2 - \log LA1)}{(t2 - t1)}$$

In the above equation, NAR is net assimilation rate (g cm^{-2} day^{-1}), whereas, w1 and w2 represent the plant dry biomass at first and final harvest, respectively, LA1 and LA2 show the leaf area at first and last harvest respectively; whereas Log represents the means of values at different harvest stages, and 't1' and 't2' were plants ages at the time of first and last harvest, respectively. Relative growth rate (g g^{-1} day^{-1}) was calculated by using the software designed by (Hunt et al., 2002).

Tomato plants were considered to have started flowering when approximately 50% of flowers on the inflorescence were fully open.

2.7 Genomic DNA Extraction

2.7.1 CTAB method

For small scale DNA extraction (15-20 samples) CTAB method was used.

2.7.1.1 Buffers and Solutions

CTAB (Hexadecyltrimethyl ammonium bromide) buffer: 2% w/v CTAB, 1.0 M NaCl, 20 mM EDTA, 100 mM Tris HCl (pH 8.0). Wash buffer: 76% ethanol, 10 mM ammonium acetate.

2.7.1.2 Extraction Method:

Freshly emerged young and tender leaves weighing approximately 75 mg were harvested and stored in 2.0 ml microcentrifuge tubes on ice until the tubes were transferred into liquid nitrogen. During this time a water bath was set up at 65^{o}C.

The tissue was ground into a fine powder using a pre-chilled Dremel drill, with care being taken to avoid thawing during grinding. 500 µl CTAB buffer pre-warmed to 65^{o}C was added to each tube and vortexed thoroughly followed by emulsification with 250 µl of each of 2% (v/v) PVP-40 and 1 %(v/v) β-mercaptoethanol. Tubes containing the mixture were incubated for 30 minutes at 65^{o}C in a water bath. During incubation, the tubes were vortexed every 10 minutes. The contents in each tube were emulsified with 500 µl Dichloromethane:isoamyl alcohol (24:1) followed by centrifugation at 13000 rpm for 10 minutes using a bench top centrifuge to separate the organic and aqueous phases. The top phase was transferred to new microcentrifuge tube followed by an addition of ice cold isopropanol.

The tubes were inverted several times to mix thoroughly followed by incubation on ice for 5 minutes and then centrifugation at 13000 rpm for 2 minutes to obtain a white compact pellet. The supernatants were carefully removed without disturbing the pellet. The pellet was washed twice by adding 500 µl wash buffer, followed by centrifugation at 13000 rpm for 3 minutes. The open tubes were left on the bench for about 5 minutes to completely dry the pellet. The pellet was dissolved in 50-75 µl of dH_2O followed by addition of 10 ng μl^{-1} *RNaseA*.

2.7.2 Qiagen DNeasy 96 well kit:

To extract DNA on large scale (more than 50 samples), Qiagen's Dneasy 96 well kit method was used according to the manufacturer's (Qiagen Ltd., UK) standard instructions.

2.8 Southern Blotting

2.8.1 Extraction of DNA for Southern blotting

DNA was extracted using Qiagen's DNeasy 96-well kit. Two or three wells of a DNeasy plate were extracted for each plant to provide sufficient DNA for Southern blotting. All DNA extractions were carried out following the 'Frozen Tissue' method from Qiagen's user manual. Typical average DNA concentration obtained through this method was about 25-35 ng μl^{-1}.

2.8.2 Restriction digestion of DNA

Restriction digestion of genomic DNA was carried out by using the restriction enzymes *Spe*-I and *Nde*-I. DNASTAR Lasergene version 8.1 was used to find the best restriction enzymes which did not cut inside the NCED gene, also the use of this combination of restriction enzymes resulted in the shortest pieces of restricted DNA fragments for improved band resolution.

Gels used for Southern blotting were 0.8% w/v agarose run in TAE (Tris-acetate-EDTA) buffer at 50 volts for approximately 16 hours followed by post staining by 1 $\mu g\ ml^{-1}$ ethidium bromide. Gels were submerged in 0.25 M HCl for 15 minutes and were gently agitated on a flat bed rocking platform. A positively charged nylon membrane (Amersham Hybond^{TM} XL), pore size of 0.45 µm and 0.4M NaOH was used

as transfer buffer. The membrane was sandwiched between two layers of 3 MM blotting sheets and incubated at 120^{o}C for 30 minutes in a vacuum oven for DNA fixation.

2.8.3 Hybridisation

Prehybridisation was carried out in a rotary incubator in a 10 cm diameter glass tube at 65^{o}C for up to 6 hours, in 30 ml of prehybridisation solution containing 2×SSPE and 0.1% (w/v) sodium dodecyl sulphate (SDS).

The DNA to be used as a probe was obtained from the restriction digestion of pNCEaa.3 plasmid. Restriction enzymes *Sac*-I and *Xba*-I were used to release the complete *LeNCED1* gene coding region of size 1.8 kbp.

The probe for hybridisation was prepared using a Random Prime DNA Labeling kit (GE Healthcare labs, UK) and standard manufacturer's protocol was followed. Hybridisation was carried out for 16-24 hours.

The hybridised blot was washed three times using the 2 × SSPE buffer (2 × SSPE,0.1% SDS w/v) followed by three washes using 1 × SSPE (1 × SSPE, 0.5%SDS) each for 15 minutes at 65^{o}C.

The blots were incubated for up to 3 days against Phosphor screens. The images were developed using a Phosphorimager (Molecular Dynamics, USA) and analysed using ImageQuant TL software.

2.9 DNA amplification and sequencing

2.9.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm the presence or absence of specific gene sequences (pimer and PCR conditions used for each reaction have been

given in Appendix-I). For a typical PCR 2.0 mM $Mgcl_2$, 0.2 mM dNTPs, 2.0 μM forward and reverse primers, 1 × PCR buffer (Invitrogen) and 0.750 units of *Taq* DNA polymerase (Invitrogen) and up to 2.0 ng of plasmid DNA and 20.0 ng genomic DNA were used. Usually the volume for each PCR reaction was limited to 10 μl. These reactions were carried out in thin walled 0.2 ml tubes or in 96-well PCR plates, in a DNA Engine DYAD Peltier Thermal Cycler (MJ Research). The PCR products were run and visualized on agarose gel (0.8 % W/V).

2.9.2 Quantitative PCR

Quantitative real-time PCR was used for the determination of T-DNA-Ds (nptII::*Ds1*::H2A::*LeNCED1*::*Ds2*) and *Tr-Ds* (*Ds1*::H2A::*LeNCED1*::*Ds2*) element copy number in various filial generations of transgenic tomato plants. SYBR Green PCR Master Mix was used to perform the assay.

Two sets of primers were designed using Invitrogen's Vector NTI Advance-10 software to amplify the *LeNCED1* gene and *Vacuolar Invertase* gene. The *LeNCED1* primers were unique to the coding region of *LeNCED1* gene. A single copy gene '*Vacuolar Invertase*' was used for the relative quantification of the *LeNCED1* gene (German et al., 2003). The reaction consisted of 2 × SYBR Green master mix (Eurogentech), 1 μM of each primer, 2 μl of DNA(25-50ng μl^{-1}) and total volume made up to 15μl by addition of dH_2O.

The plate was incubated in an ABI Prism 7900HT Sequence Detection System at default profile settings (50°C for 2 minutes; 95°C for 10 minutes followed by 40 cycles of 95°C for 15 sec and 60°C for 1 minute). For each plant three biological and two technical replicates were used.

Where possible, a plant with known zygosity was included in the assay for the estimation of the copy number using the Threshold Cycle (Ct) values. Ct is defined as the point during the PCR where the fluorescence from SYBR Green reaches the threshold level. To calculate the relative gene copy number the following formula was used (German et al., 2003);

Relative gene copy number =

$$2^{(\text{Ct reference gene-Ct transgene})}/2^{(\text{Ct reference gene of control-Ct transgene in control})}$$

2.9.3 Reverse Transcriptase PCR

Reverse transcriptase (RT-PCR) was used for the quantification of *LeNCED1* mRNA in different genotypes at various growth stages. Total RNA was extracted from leaves by using RNeasy kit (Qiagin, UK, ltd.). This RNA was treated with DNaseQ to avoid any DNA contamination. The DNA free RNA was converted into cDNA by using Superscript II (Invitrogen), random hexamers were used as primers in this reaction. The RNA (0.5μg) and random hexamers (200 ng) were heated to 65^{o}C for 10 minutes followed by cooling to room temperature then placed on ice for 5 minutes. This was followed by adding 100U Superscript II, 0.5 mM dNTPs, 5mM DTT and 1×First Strand Synthesis buffer (Invitrogen) to the final concentration in total volume of 20 μl. The reaction was carried out by incubation at 42^{o}C for 90 minutes. This was followed by 75^{o}C for 10 minutes. Assuming that 100% RNA was converted to cDNA the contents of this reaction was diluted to 5 folds to get 5 ng μl^{-1} final concentration.

The PCR conditions were used as described in section 2.9.2. The reaction consisted of 1.0 μl of 20×SYBR Green Master mix (Eurogentic) 1 μM of each primer and 10 ng of cDNA (2 μl) to a total volume of 20 μl. Each reaction was performed with

three biological and two technical replications. To perform a control negative reaction, no cDNA template was added to test for contamination from chromosomal DNA, 10 ng of DNase treated RNA was used. The cDNA from a random genotype was used to generate standard curves using up to seven folds dilutions. The *Le18S* rRNA primers were designed by Dr. Martin Sergeant (Research Scientist) WHRI, University of Warwick.

2.9.4 Sequencing

Sequencing reaction of Empty Donor Site (EDS) was carried out by using BigDye (Applied Biosystems, USA). Approximately 10 ng of template DNA were added to a reaction mixture with 2 μl BigDye terminator version 3.0, 1×BigDye dilution buffer and 1.0μl (final concentration 2μM) primer specific to the probe.

A total reaction volume of 10 μl was incubated at 94^{o}C for 3 minutes followed by 25 cycles at 96^{o}C for 10 seconds, 50^{o}C for 5 seconds and 60^{o}C for 4 minutes. The sequencing was performed by using Applied Biosynthesis 3130×l DNA Analyser in Genomic Centre at WHRI. The results obtained were analysed by using MegAlign (DNASTAR Inc. USA) software.

2.10 Statistical Analysis

For plant stomatal conductance data collected under control environment, randomized complete block design (RCBD) was used and its analysis was carried out using Genstat v.9. For phenotypic ratios and linkage analysis Chi-square test was used. To compare the means, Students T-Test was used assuming equal or unequal variance as

required. For sequential harvest experiment, the averages were calculated across each block and data was analysed by using analysis of variance (ANOVA).

Chapter-3 Activation and transposition of an *LeNCED1* transgene

3.1 Introduction

In this study a two-way transposon-based transformation system was adapted with the aim of achieving an expression of the *LeNCED1* gene to get an optimum level of phytohormone ABA required for reduction in the plant stomatal conductance without having any effects on plant growth and establishment.

Table 3-2: Important acronyms used in the text regarding the transposons

Acronym	Detail
T-DNA	The transferred-DNA which has been integrated into the plant genome following transfer from the Ti-plasmid during *Agrobacterium*-mediated transformation (*LB::nptII::Ds1::pH2A::LeNCED1::t35s::Ds2::RB*). LB, left border; RB, right border; t35s, CaMV 35s transcription terminator.
Ds element	The term used in general for the *Ds* element which may occur either within the T-DNA, or elsewhere in the genome following transposition (*Ds1::pH2A::LeNCED1::t35s::Ds2*).
T-DNA-Ds element	The *Ds* element which remains within the T-DNA and that has not undergone transposition. It therefore remains linked to the kanamycin resistance gene. Same structure as "*Ds* element"
Tr-Ds element	The transposed *Ds* element: the part of the T-DNA-Ds element which transposed out of the T-DNA-Ds element and reintegrated in the genome leaving behind the kanamycin resistance gene and left and right borders. Same structure as "*Ds* element"

3.1.1 The constructs *pBP6* and *sAc* and their transformation into tomato:

The construct *pBP6* was prepared by Mrs Alison Jackson in Dr. Andrew Thompson's lab at Warwick HRI (Defra project HP0218). In this construct, the *LeNCED1* gene was flanked by the border sequence of the maize *Ds* element to give *Ds1*::pHistoneH2A::*LeNCED1*::*Ds2* (Figure 3.1). A histone H2A promoter from tobacco was used to drive the expression of the *LeNCED1* transgene because of its low constitutive expression. The Histone promoter H2A was originally thought to be root specific but was later discovered to have mild constitutive expression in both roots and shoots (Jones, 2007). The constitutively mild expression is desirable in this study as the transposition of the *Ds* element carrying the histoneH2A promoter near to an enhancer region may increase transgene (*LeNCED1*) expression to various extents and tissue-specific patterns. The construct pBP6 was transformed into tomato (Ailsa Craig $Tm2^a$) plants at Warwick HRI in Dr. Andrew Thompson's laboratory and tomato lines Ds517-1 and Ds517-7 (T_2 seed accessions AT94, AT103, respectively) were selected for further work in this project because they had apparently normal growth and development, and exhibited 3:1 segregation of kanamycin resistance in the T_1 generation which indicates that they contained the pBP6 T-DNA in a single locus.

The construct containing the stabilised activator (*sAc*) was previously designed (Jones et al., 1992) and is shown in Figure 3.2. This construct carries an *Ac*-derived structure having 3′ deletions in its TIRs. Due to these deletions, the *Ac* element cannot transpose and becomes a stablised *Ac* element (s*Ac*). But the deletion in the 3′ end does not impair the ability of the *sAc* element to provide the transposase (*Tpase*) which is necessary for the activation of the *Ds* element (Carroll et al., 1995).

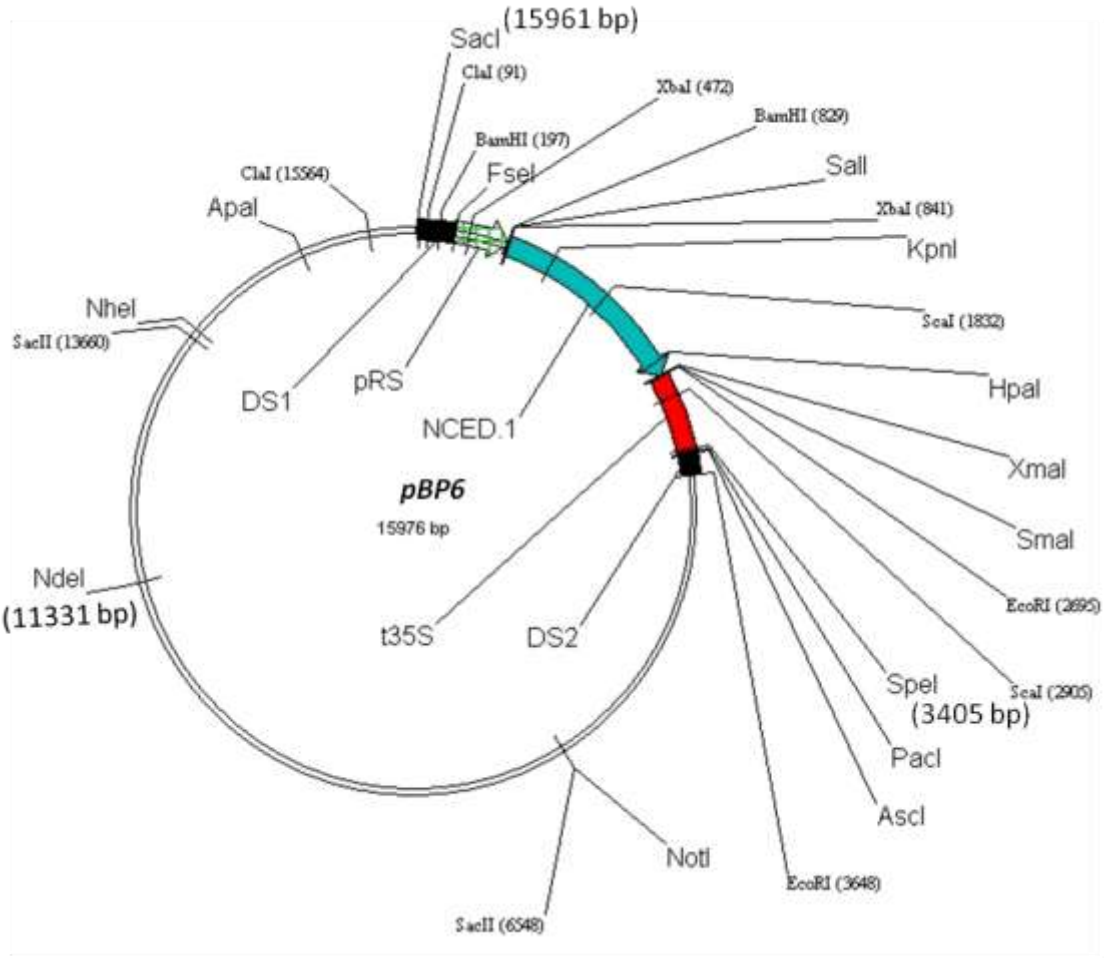

Figure 3-1. The *pBP6* construct containing the *Ds1*::p*H2A::LeNCED1::t35s::Ds2* element**.** Where: *Ds1* and *Ds2*, Terminal Inverted Repeats (Rubin et al.) obtained from maize *Ds* element; pRS, Histone H2A promoter (previously believed to be root specific) sequence from tobacco; NCED1, open reading frame from tomato gene 9-*cis*-epoxycarotenoid dioxygenase; t35S, terminator sequence from CaMV.

This construct was transformed in tomato (Moneymaker) in Prof. Jonathon Jones' lab. The construct was named JJ-T100 and the subsequent transformed seed accession was named as AT1195 according to the nomenclature used for new accessions by Dr. Andrew Thompson's lab. The *sAc* was linked with the β-glucuronidase (GUS) reporter gene. In order for the *Tr-Ds* element to be stabilised in the genome, plants must

not contain the *sAc* element. Plants lacking *sAc* can be selected as those which have lost the GUS gene and associated activity (GUS negative plants) through segregation.

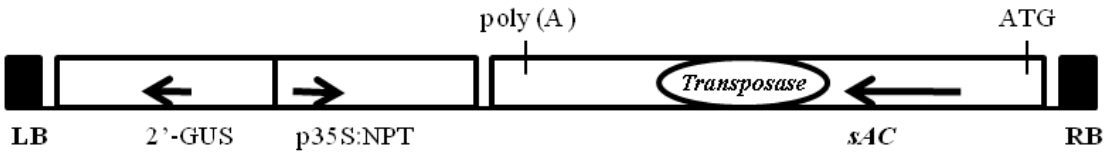

Figure 3.2. The *sAc* construct JJ-T100. LB and RB, Left and right borders, respectively; 2'-GUS, GUS gene driven by 2' promoter; p35S, CaMV 35S promoter; NPT, neomycin phosphotransferase II gene; *sAc*, stabilised activator element; poly(A), polyadenylation sequence; ATG, start codon sequence. Arrows indicate the direction of transcription. (Jones et al., 1992).

3.1.2 Activation of T-DNA-Ds element to transpose *Tr-Ds* element in transgenic tomato lines

It is important to mention that the tomato genome does not contain its own active *Ac/Ds* transposon system (Emmanuel and Levy, 2002) which could influence the activity of transformed transposable elements. So after screening of GUS containing plants it was possible for the *Tr-Ds* element to stably integrate in the genome and could be inherited to the next generation.

The T_2 progeny of tomato lines Ds517-1 and Ds517-7 genotyped for the presence of T-DNA-Ds elements were each cross pollinated with the plant containing *sAc* element (construct JJ-T100, accession AT1195) so that the *Ds* element could be activated in the presence of *sAc* element in the F_1 generation. The crosses between the two transgenic lines were performed at the University of Nottingham by Dr. Ian Taylor. An illustration of the detailed scheme of the two component transposon system is given in Figure 3.3.

The F_1 seeds resulting from the cross between the lines containing the T-DNA-Ds and *sAc* elements did not germinate when imbibed on water alone (Ian Taylor, pers. comm..), but some did germinate on norflurazon (3.3 μM), an inhibitor of ABA biosynthesis (germinated by Howard Hilton, WHRI). At the beginning of this PhD project, three F_1 seedlings were available for study in which transposition of the *Tr-Ds* element should be occurring. These plants were:

Ds517-1 T_2 × JJ-T100 F_1 (from seed accession AT1196)

Ds517-1 T_2 × JJ-T100 F_1 (from seed accession AT1197 plant-A = AT1197-A)

Ds517-1 T_2 × JJ-T100 F_1 (from seed accession AT1197 plant-B = AT1197-B)

As these crosses involved only Ds517-1 T_2 line, hence, the Ds517-7 line was excluded from further experimental work (detail of seed accessions present in Appendix-III).

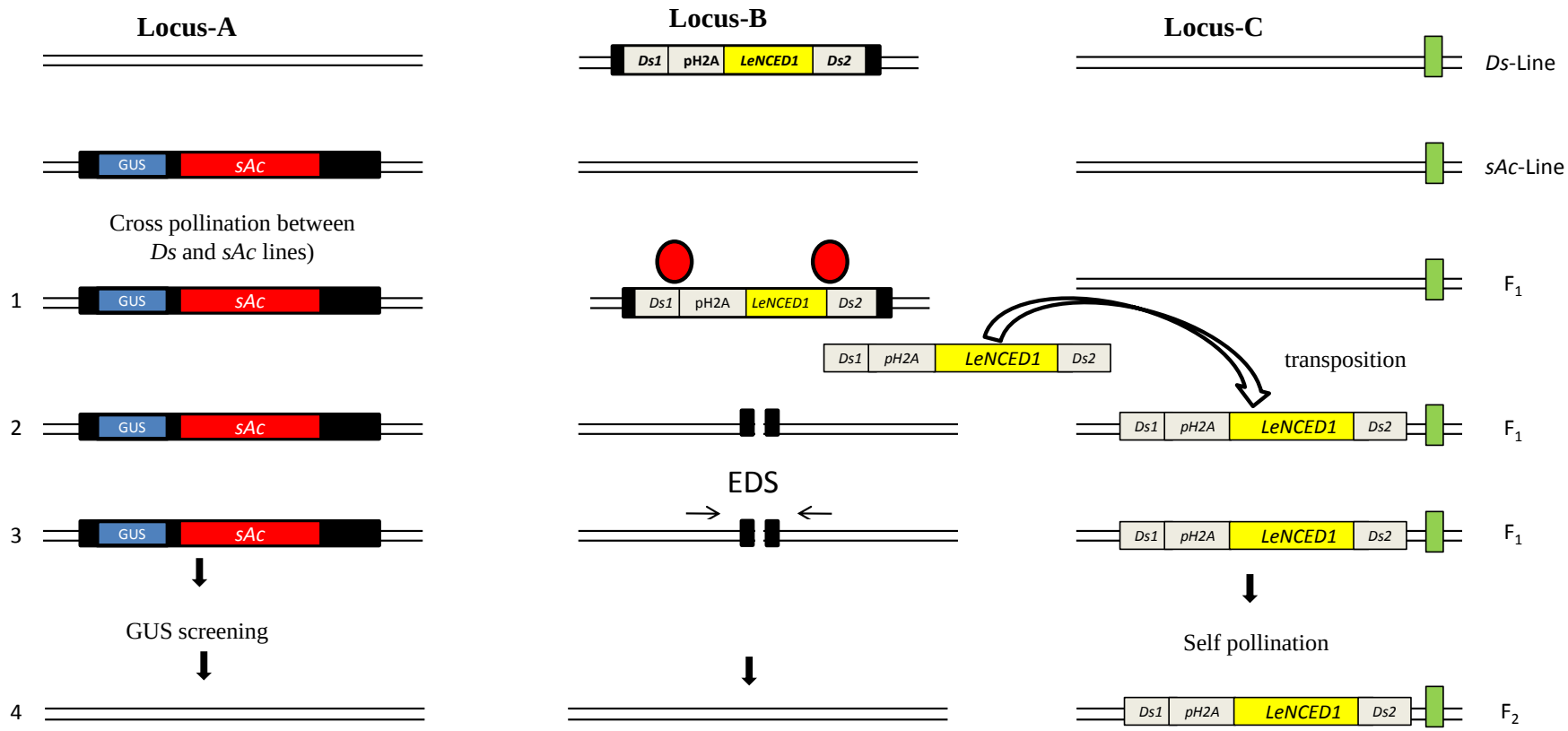

Figure 3.3 A schematic diagram of approaches employed to achieve objectives of transposition. 1.Cross pollination of *sAc* and *Ds* lines 2.Transposase activates the *Ds* element; 3. *TrDs* element transposes in the genome and leaves EDS 4. Segregation of *sAc*, its screening out, self pollination of F_1 plants to achieve F_2 progeny. *sAc*= stabilized activator line *Ds* line= Plant containing *LeNCED1* gene EDS= Empty Donor Site TIR= Terminal Inverted Repeats GUS= β-Glucuronidase for *sAc* screening Left and Right Borders Pimer Pair ⇆ pH2A= Histone H2A promoter Transposase Enhancer region

3.2 Results

3.2.1 Confirmation of the presence of *sAc* and *Ds* element in F_1 plants from the cross (Ds517-1 T_2 x JJ-T100)

To confirm the presence of the *Ds* element (T-DNA-Ds or the *Tr-Ds* element) in the F_1 plants, primer pair *Ds1*For2 and *notRev5* (Appendix-I) were used to amplify a region spanning *Ds1* and the *LeNCED1* open reading frame (ORF) presented in Figure 3.4.A. The predicted band size was 839 bp and the gel image (Figure 3.4-B) confirmed the presence of the T-DNA-Ds or *Tr-Ds* element in two of the three F_1 plants. Because one of the F_1 plants lacked the T-DNA-Ds or *Tr-Ds*, it was concluded that the parent *Ds* plant (Ds517-1 T_2) was heterozygous for the T-DNA-Ds element and therefore only 50% of the F_1 progeny would be expected to contain the T-DNA-Ds

As the *sAc* construct contains a functional GUS reported gene, the F_1 plants could be tested for the presence of the *sAc* T-DNA using a GUS activity assay. The excised roots from all three F_1 plants were found to give a blue colour during the GUS assay.

3.2.2 Detection of empty donor site in F_1 plants

The F_1 plants were tested by PCR for the empty donor site (EDS) to determine if *sAc* element had activated the *Ds* element. As previously described in section 3.1 the *sAc* element provides *Tpase* which is necessary for the activation of the *Ds* element. On excision of the original T-DNA-Ds element by the *Tpase*, the EDS remains, consisting of the nptII gene and the left and right borders only. If there was no transposition of the *Ds* element, only an amplicon of 4279 bp could be produced in F_1 plants using the primer pair *DsNCED1For2* (15901 bp-15918 bp) and *pBP6*(RS)Rev1 (4186-4203) or

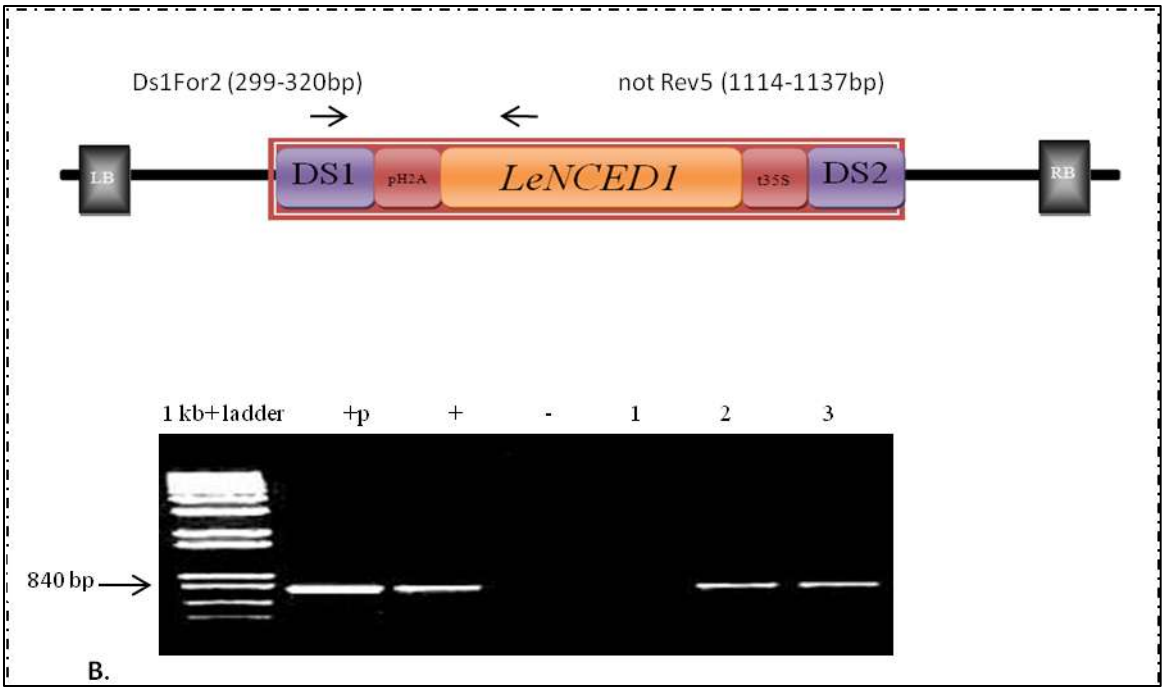

Figure 3.4. Amplification of *Ds* (T-DNA-Ds/Tr-Ds) element in F_1 plants. **A**. Plasmid pBP6 showing the regions of amplification of the *Ds* element by the primers used in panel (not to scale). Arrows indicate the direction of primer extension. **B.** Agarose gel image of PCR products. Plasmid pBP6 (+p) and Ds-517-1 line (+) were used as a positive control. Where wild type as negative control (-) and lines 1-3 represent the individual F_1 plants: 1, AT1196; 2, AT1197-A; 3, AT1197-B. The Primers used were *Ds1For2* and *notRev5*.

3844 bp by using primers *DsNCED1For2* (15901 bp-15918 bp) and *EDSRev2* (3749-3768 bp) illustrated in Appendix-II. Contrary to this, a successful activation of the T-DNA-Ds element would result in a much smaller (646 bp) amplicon from the EDS (Figure 3.5-A, B).

The PCR results of the positive control (plasmid pBP6) shown in Figure 3.5-C showed an amplification product of 4279 bp when the primers *DsNCED1For2* (15901 bp-15918 bp) and *pBP6*(RS)Rev1 (4186-4203), detailed in Appendix-I, were used. While using the same primer pair with DNA from F_1 plants as template, only a band

consistent with a size of 646 bp was amplified, indicative of the presence of the EDS. Since no transposition is possible in the preparation of plasmid, it is not expected to have the EDS, only a band of 4279 bp could be produced (Figure 3.5-C). After changing the PCR conditions it was possible to amplify both bands (4279 bp and 646 bp) in F_1 plants (Figure 3.5-D), the PCR conditions are described in Appendix-I. Again these results confirmed the presence of EDS in both F_1 plants, although in the AT1197-A plant only DNA from one leaf sample contained EDS, while the second sample obtained from a different shoot did not contain the EDS.

3.2.3 Sequence analysis of empty donor site

In order to confirm that the ~646 bp band amplified during the PCR did contain the DNA from EDS, these bands were extracted from the gel and sequenced (Figure 3.6). In Figure 3.6-A, complete sequence of the T-DNA-Ds element in the region amplified by the *DsNCED1For2* and *pBP6(RS)Rev1* primers is given, whereas Figure 3.6-B shows the sequence results from amplification of the putative EDS. SeqMan (Lasergene version 8 DNA Star inc. Madison, Wisc.) was used to align all the sequences obtained from the DNA extracted from the gel. The sequencing results were not of high quality because they showed large regions of "mixed" sequence and only small regions of readable sequence. However, the results suggested that the *Ds* element inside the T-DNA had transposed because sequences flanking the *Ds* element within the T-DNA were present at each end of the ~646 bp fragment (Figure 3.6).

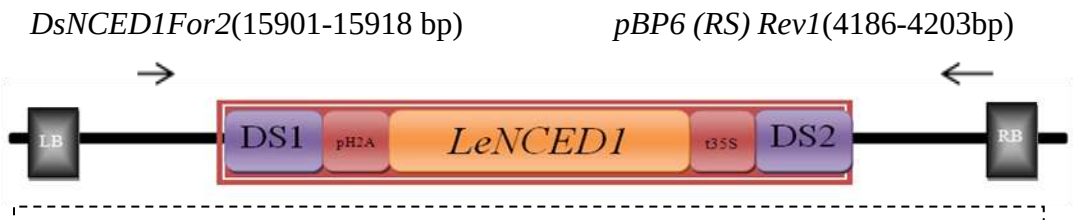

Figure 3.5. Detection of empty donor site in F_1 plants. **A.** Amplification of 4279 bp band using the primers DsNCED1For2 and *pBP6*(RS) Rev1 in plasmid *pBP6*.

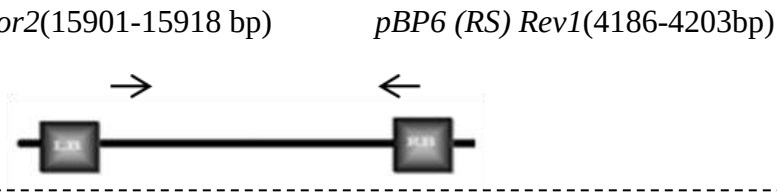

Figure 3.5-B. The transposition of Ds1::histoneH2A::*LeNCED1*::Ds2 element resulted in only 646bp in F_1 plants.

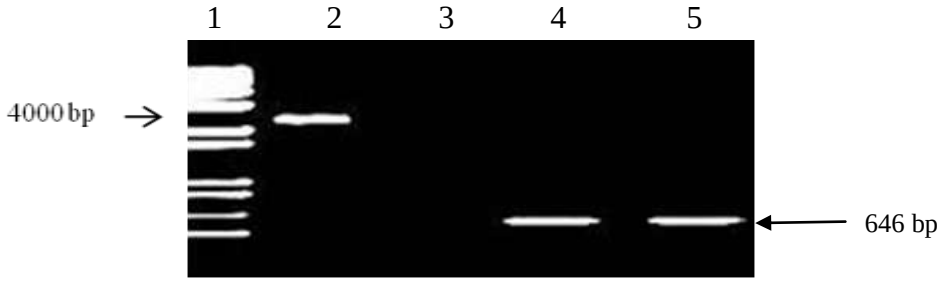

Figure 3.5-C. Ethidium bromide-stained agarose gel electrophoresis image of PCR products from F_1 plants. 1, Kb+ DNA ladder; 2, plasmid (*pBP6*); 3, wild type; 4, AT1197-A; 5, AT1197-B.

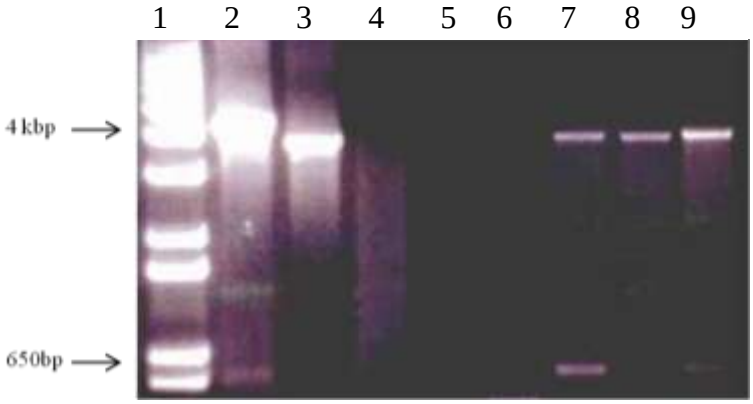

Figure 3.5-D. Ethidium bromide-stained agarose gel electrophoresis image of PCR products from F_1 plants. 1, Kb+ DNA ladder; 2, plasmid (*pBP6*); 3, Ds717-1; 4, wild type; 5, Water only; 6, AT1196; 7, AT1197-A(A); 8, AT1197-A (B) 9, AT1197-B. Where 'A' and 'B' represent two separate DNA samples from leaf tissues.

It is postulated that the excision of *Ds element* from the T-DNA of the F_1 plants had been imprecise and this resulted in a PCR product that was a mixture of several slightly different sequences that therefore resulted in a sequence chromatogram consisting of several superimposed sequences that were difficult to read. Therefore a *Topo TA PCR* cloning kit (pCR II-TOPO vector, Invitrogen) was used in an attempt to clone individual EDS sequences, however this was unsuccessful.

A.

5’ **agctggcgcgga**gctcagggat……4279bp…… atccctagatagaattc**attaacaatt**cactggccgtcg-3’

5’ cc**agctggcgcgga**nnntn……646 bp……**attaacaatt** n**actggccgt**ngttnnnn c **-3’**

B.

Figure 3.6. Sequence results of the empty donor site from F_1 plants (Ds517-1 x JJ-T100). **A.** The flanking sequence of the T-DNA-Ds element from pBP6 in the range of DsNCED1For2 and *pBP6*(RS)Rev1 primers. **B.** the equivalent sequence obtained from the 646 bp band from the F_1 plants (as detected in Figure 3.5-C)

3.3 Discussion

3.3.1 Transposition as a novel tool for generation of variation in plants

The two component transposon-based system used in this study is a novel tool with the potential to cause random variation in the expression of an *LeNCED1* transgene in tomato plants. Here, we used an *Ac* element from maize, originally discovered by Barbara Mclintock, 1947, incapable of excision (s*Ac*) but it could contribute towards the transposition of the *Ds* element (Conrad and Brutnell, 2005, Jones et al., 1989).

Many studies have demonstrated the successful transposition of *Ds element*s during the F_1 generation in the presence of an *Ac* element in various crops (e.g. in

A.thaliana (Grevelding et al., 1992)), tobacco (Fitzmaurice et al., 1999), and tomato (Spassieva et al., 1998) but the aim of most of these studies was gene discovery through insertional mutagenesis or gene tagging. The present study is unique as the objective of the study was to use an engineered *Ds* element for transposition of a transgene to random positions in the genome to generate variation in expression of the transgene.

3.3.2 Successful activation of the *Ds* element

Cross pollination of plants Ds517-1 and JJ-T100 (s*Ac* x pBP6) brought the two transposable elements (s*Ac* and T-DNA-Ds) together which promotes the transposition of T-DNA-Ds elements to integrate *Tr-Ds* elements in new locations of the tomato plant genome. Most of these excision events are thought to be germinal (Greco et al., 2003) and inheritable (Altmann et al., 1995, Grevelding et al., 1992, Li et al., 2008). A good and easy way to detect successful transposition in plants is the presence of EDS. As a result of excision of the T-DNA-Ds element an EDS was produced, similar approach was adapted by (Belzile et al., 1989) while working on tomato and (Murai et al., 1991) during their study on maize crop to amplify EDS and confirm the transposition of the *Ds* element in these plants. Since the EDS was detected in the F_1 plants in this chapter, it is clear that the transposon system used here was active in excision, and by inference also in the generation of newly integrated *Tr-Ds* elements in new locations.

Whereas the absence of EDS in one of the DNA samples obtained from F_1 plant AT1197-A show in Figure 3.5-D can either be a result of this particular plant tissue arisen from a cell lineage with no transposition events or it might be low molar concentration of EDS in this DNA sample which could not produce detectable band on the gel.

Several studies have reported that the excision in maize transposable elements is imprecise (Baker et al., 1986). On insertion to a new site in the genome in maize, *Ac* elements resulted in 8-bp duplication (Ohare and Rubin, 1983, Long et al., 1993). On reactivation it leaves behind a part or all of its duplication which can be exactly the same or slightly modified which could be due to imprecise excision of the *Ds* element (Hehl and Baker, 1989). This excision resulting in *Ds* element sequences remaining at the target site might be due to random double stranded cleavages at the junction resulting in imprecise restoration of original target sequence (Voelker et al., 1984, Obrochta et al., 1991). This explains why regions of "mixed" sequences were obtained in Figure 3-6.

3.3.3 Transposed *Ds* element might be a source of over accumulation of ABA in F_2 (Ds517-1 T_2 × JJT100) seeds

The transposable elements (*Ac/Ds*) can potentially generate a wealth of genotypic and phenotypic variation in plants on their insertion into new positions in the genome. This ability of transposons was utilized by many researchers (Kuromori et al., 2004, Park et al., 2007). However, the approach adapted during the present study was completely different where the *Ds* element carried a functional gene so that its transposition to different parts in the genome could have changed the expression of the transgene (*LeNCED1*) present in the *Ds* element.

In previous studies the overexpression of *LeNCED1* using the Gelvin super promoter (sp), it was observed that the construct (sp:*LeNCED1*) resulted in plants with high ABA contents and the resultant seeds failed to germinate when incubated on water alone (Thompson et al., 2000). The lack of germination in the F_1 seeds in this experiment may have been an early indication of transposition of *Tr-Ds* element containing the *LeNCED1* transgene in F_1 plants.

Hence, it can be postulated at this stage that the overaccumulation of ABA in the germinating embryo might have caused the seed dormancy (Thompson et al., 2000, Qin and Zeevaart, 2002) resulting in poor seed germination. Further, it was observed that one of the two F_1 plants (AT1197-A) containing the T-DNA-Ds element had spindly stem with slower growth and establishment with poor fruit set and boxy fruits with very little seeds inside. This change in plant phenotype could be attributed to the insertional mutation caused by the *Tr-Ds* element (Long et al., 1993, Weck et al., 1984), or to a great excess of ABA in the plant interfering with normal plant growth and development.

The F_2 seed obtained from self pollination of F_1 plants was used to screen the variation in phenotypes that may result from variation in ABA biosynthesis rates caused by transposition of the *LeNCED1*-containing *Ds* element. These screens are described in Chapter 4.

Chapter-4 Screening for variation in stomatal conductance in the F_2 generation of the cross *sAc* × *Ds*

4.1 Introduction

4.1.1 *Ac/Ds* transposition system: A source of genetic variation in plants

Transposition of *Ds* elements is generally random in the plant genome but it is preferential for reintegration into, for instance, the regions rich in 'GC' content or near the promoter regions of a gene (Vanschaik and Brink, 1959, Greenblatt and Brink, 1962, Moreno et al., 1992, Tardieu and Davies, 1992). The integration of the *Ds* element to create a transposed *Ds* can produce a remarkable diversity in plant phenotypes by changing the host (plant) gene expression (Coen et al., 1986). If the *Tr-Ds* element inserts into an intron, it can change the RNA processing pattern by the alteration of the host gene splicing site choice (Mount et al., 1988); while, insertion of the *Tr-Ds* element into a coding region would most likely result in the disruption of protein function (Kidwell and Lisch, 1997).

A successful reintegration of the *Tr-Ds* element carrying the transgene *LeNCED1* near a transcriptionally active site (Parinov et al., 1999, Meissner et al., 2000) can result in enhanced expression of the transgene (*LeNCED1*), hence an increased biosynthesis of ABA (Thompson et al., 2000, Qin and Zeevaart, 2002) which can affect plant growth and development in many different ways including reduction in the plant stomatal conductance (g_s) (Tardieu et al., 1992, Wilkinson and Davies, 2002), seed germination (Thompson et al., 2007, Lopez-Molina et al., 2001) and alterations in plant growth and biomass production (Zhang and Davies, 1990, Sharp et al., 2000).

4.1.2 Different approaches to measure leaf g_s

The g_s can be quantified by different means, including Porometer, Infra Red Gas Analyser (IRGA) and infra red thermal imaging. For efficient g_s screening, a rapid and robust technique is crucial as the g_s is sensitive to a small change in CO_2 concentration, vapour pressure deficit (VPD) and light (Jarvis, 1976). The steady state diffusion Porometer can provide rapid assessment of leaf g_s (Schulze et al., 1982) under the natural environment of a large number of leaves, hence minimising the risk of drastic changes in the environmental conditions.

In Chapter 3, it was demonstrated that the transposons, *sAc* and *Ds*1::H2A::*LeNCED1*::*Ds*2 were active in the F_1 generation indicated by the presence of empty donor site. Further, the increased seed dormancy in the F_2 generation might also be due to the transposition of the *Tr-Ds* elements to transcriptionally active sites in the genome.

This chapter contains the detail about screening of large number of GUS negative *sAc*×*Ds*-F_2 plants with stable integration of the *Ds* element and its effects on seed germination, plant growth and g_s.

4.2 Results

4.2.1 Obtaining *sAc* × *Ds*-F_2 seed

The *sAc*×*Ds*-F_1 lines containing the T-DNA-Ds, *sAc* and potential *Tr-Ds* elements were self-pollinated to get the F_2 progeny. It was observed that in one of the F_1 lines (AT1197-A), there was poor fruit set and most of the fruits were boxy in shape with fewer seeds inside compared to the fruits obtained from wild type plants (Figure 4-

1).

Figure 4-1. Fruit shape and seed set in F_1 plants. Top, a cross section of wild type fruit, Bottom a cross section of fruit from AT1197-A plant, note fewer seeds and boxy shape.

To improve the fruit set, cuttings were taken from the side shoots of the AT1197-A F_1 plant and propagated to obtain several plants. These plants were then reciprocally backcrossed with Ailsa Craig $Tm2^a$ (WT) to give BC_1F_1 seed, or they were allowed to self-pollinate to give F_2 seed. Through this approach a large number of F_2 seed (self pollination of AT1197-A) and BC_1F_1 seeds were obtained for future experimental work.

Backcrossing has two further advantages: (i) to reduce the effect of somaclonal variations, if any, that might have arisen during tissue culture of the transformed plants (Bregitzer et al., 2008) and that might have conceivably contributed towards a reduction in g_s, and (ii) to eventually allow a well-defined genetic background to be achieved. The genetic background of the *sAc* parental line was Moneymaker and the background for the *Ds* line was Ailsa Craig $Tm2^a$ and thus the F_2 will segregate for any polymorphisms between these two parents, some of which could possibly influence g_s.

4.2.2 Germination and establishment of *sAc* × *Ds*-F_2 seedlings

The *sAc*×*Ds*-F_2 and wild type seeds (accession AT1661) were germinated using the hydroxamic acid D4 (1.0 mM), a chemical inhibitor of NCED (Sergeant et al., 2009), so that the seeds containing more ABA than the wild type could still germinate; whereas, sp5 (control) seeds were germinated using 3.3 µM norflurazon, also to inhibit ABA biosynthesis and so to promote germination. Norflurazon is a strong inhibitor of ABA biosynthesis in plants. It inhibits *phytoene desaturase* by competing with the cofactor required for enzyme activity (Breitenbach et al., 2001). D4 is also an ABA inhibitor but its target enzyme is NCED. This inhibitor is less potent and only results in improved germination of genotypes containing moderately increased seed ABA (detail present in Chapter-7) (Awan et al., unpublished). The advantage of using D4 over norflurazon was that it did not cause any photobleaching of the cotyledons. The use of a very strong inhibitor of ABA biosynthesis to stimulate germination in this screen is of no great advantage because the aim of the screen is to find lines with normal germination and high WUE. Upon germination, seedlings were transferred to rockwool blocks where the plants remained for up to two-weeks in a hydroponic system utilizing the Nutrient Film Technique. The NFT was used to allow easy access to root tissue for GUS screening without damaging the plant root system.

A large variation was recorded in the rate of seed germination, as determined by radicle emergence (Figure 4-2) of *sAc*×*Ds*-F_2. It was also visually apparent that there was a large variation in seedling emergence, establishment and plant growth rates, although data was not collected. It was also noted that 8.0% (28 out of 351) of *sAc*×*Ds*-F_2 seedlings containing the *Ds* element confirmed through PCR (see below for detail)

had a characteristic phenotype consisting of narrow, 'needle-like' leaves, pale green foliage, slow growth and poor fruit set (Figure 4-3).

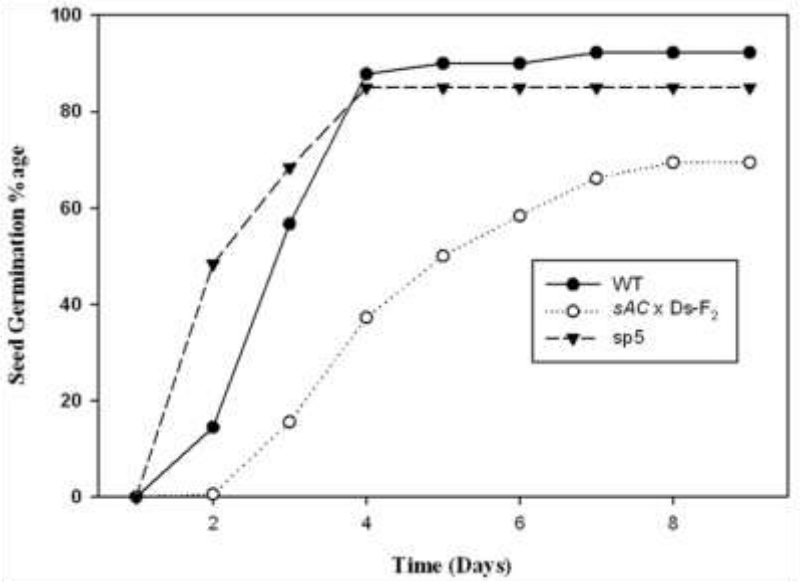

Figure 4-2. Rate of seed germination in *sAc* × *Ds*-F_2 seed used during the plant screening programme. The wild type (Ailsa Craig $Tm2^a$) and sp5 were used as control genotypes. The sp5 seeds were treated with 3.3 μM norflurazon overnight, the wild type and *sAc* × *Ds*-F_2 seed (accession AT1661) were treated with 1.0 mM D4 overnight.

Figure 4-3. The *sAc*×*Ds*-F_2 plants showing abnormal 'needle like' leaves. Plants were grown on Nutrient medium on rockwool plugs in peat-based compost. Plants marked as 'A' and 'B' showed signs of a phenotype with 'needle like' leaves. The image was taken from 18 days old plants

4.2.3 GUS screening of *sAc*×*Ds*-F_2 plants to eliminate the *sAc*

The *sAc* gene was linked to the β-glucuonidase (GUS) gene (Thomas et al., 1994). The cross pollination of the plant containing the *sAc* element with the plant containing the T-DNA-Ds element resulted in all three of the *sAc*×*Ds*-F_1 progeny being GUS positive. The *sAc* element activates the *Tr-Ds* element (Carroll et al., 1995), hence in the presence of *sAc* (indicated by GUS activity), *Tr-Ds* element cannot be stabilised in the genome. The *sAc*×*Ds*-F_2 plants which gave root samples that did not turn blue (Figure 4-4) in the presence of X-gluc were selected because they should contain stabilised *Ds* element(s) present in the genome.

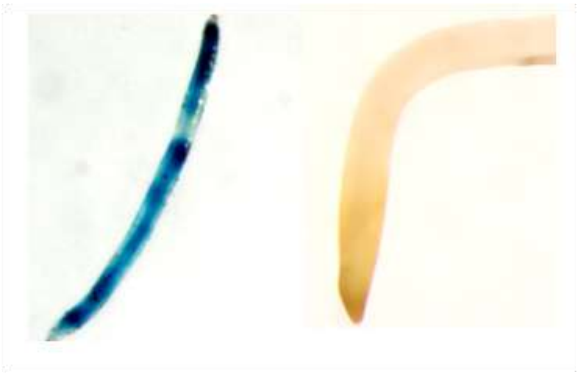

Figure 4-4 Screening for GUS reporter gene in *sAc*×*Ds*-F_2 plants. GUS Positive root sample (Left) GUS negative on the right.

In total, 1695 F_2 plants were tested for GUS activity by taking a section of root and performing a stain with X-Gluc. The results revealed that 73.7% (1249/1695) of *sAc*×*Ds*-F_2 progeny tested positive for the GUS reporter gene (Table 4-1). The GUS negative *sAc*×*Ds*-F_2 plants were then subjected to PCR, the primers used for the confirmation of *Ds element* present in *sAc*×*Ds*-F_2 plants were *Ds1For2* and *not Rev5.*

Table 4-1: Segregation ratio in the *sAc* × *Ds*-F_2 generation

Genotype	Expected ratio**	No.of plants expected	No.of plants observed	$x^2_{calc.}$	$x^2_{0.05,3}$	Ratio 9:3:3:1
GUS positive (*Ds* unknown)*	9+3=12	1271.3	1249	4.97	7.82	Yes
GUS negative, *Ds* positive	3	317.8	351			
GUS negative, *Ds* negative	1	105.9	95			
Total	16	1695	1695			

*The *sAc*×*Ds*-F_2 plants containing the GUS reporter gene were discarded; hence the *Ds* genotype of these plants was not determined. **assuming Mendelian independent segregation of the *sAc* and a single *Ds* locus.

The PCR results confirmed that 20.7% (351/1695) of total *sAc*×*Ds*-F_2 plants contained the *Ds* element only. These results were analysed (Table 4-1) using a Chi-Square test giving $P > 0.05$, indicating that the ratio was not significantly different from the ratio of 12:3:1 which would be expected if the two loci (s*Ac* and T-DNA-Ds) were segregating independently, and no unlinked *Tr-Ds* elements were formed. However, data in Table 4-1 showed that the GUS negative plants containing the *Ds* element were relatively higher (351) compared to the expected plants (317.1), although it did not alter the inheritance ratio in the *sAc*×*Ds*-F_2 plants.

4.2.4 Screening *sAc*×*Ds*-F_2 plants for reduced stomatal conductance

During this screening programme, GUS negative *sAc*×*Ds*-F_2 plants containing the *Ds* (T-DNA-Ds/*Tr-Ds* elements) were selected as described above. Plants containing the T-DNA-Ds and potentially *Tr-Ds* element (*Ds* elements) were used to screen for reduced g_s. The *sAc*×Ds-F_2, wild type and sp5 seeds were sown at different time intervals to synchronize the germination and size of the plants. These were the same seeds used for the germination assay described above. Seeds of genotype sp5 were sown 5-7 days earlier than the wild type or *sAc*×*Ds*-F_2 seeds as the high ABA genotype sp5 was slow to establish (Thompson, 2007). The *sAc*×*Ds*-F_2 seedlings with poor establishment and any growth defects were discarded at this stage because the intention was to obtain lines with good growth characteristics but reduced g_s.

Approximately 40-42 day old plants were transferred to a walk-in controlled environment cabinet to measure the g_s. These plants were irrigated to field capacity and left for at least one hour to acclimatize in the control environment prior to g_s data collection. Plants were grouped in randomized blocks each containing wild type, sp5

and *sAc*×*Ds*-F_2 genotypes. Data was collected from a single standardised leaf from each plant four times a day using a handheld Porometer. During this study, more than 250 GUS-negative *sAc*×*Ds*-F_2 plants containing the *Ds* element(s) were screened for their g_s.

The *sAc*×*Ds*-F_2 plants with g_s values higher than or similar to wild type were not selected (Figure 4.5 F_2-NS), whereas, the *sAc*×*Ds*-F_2 plants with lower g_s than wild type were chosen (Figure 4.5, F_2-S) for seed bulking to obtain *sAc*×*Ds*-F_3 progeny. The backcross progeny (BC_1F_1) from *sAc*×*Ds*-F_1 plants (Ailsa craig $Tm2^a$ x *sAc*×*Ds*-F_1 was also tested for its g_s in the controlled environment (Figure 4.5, screen 5&6). Results in screen 5 and 6 revealed that the backcrossing increased the g_s in BC_1F_1 plants compared to the lines generated through selfing of the F_1 plants.

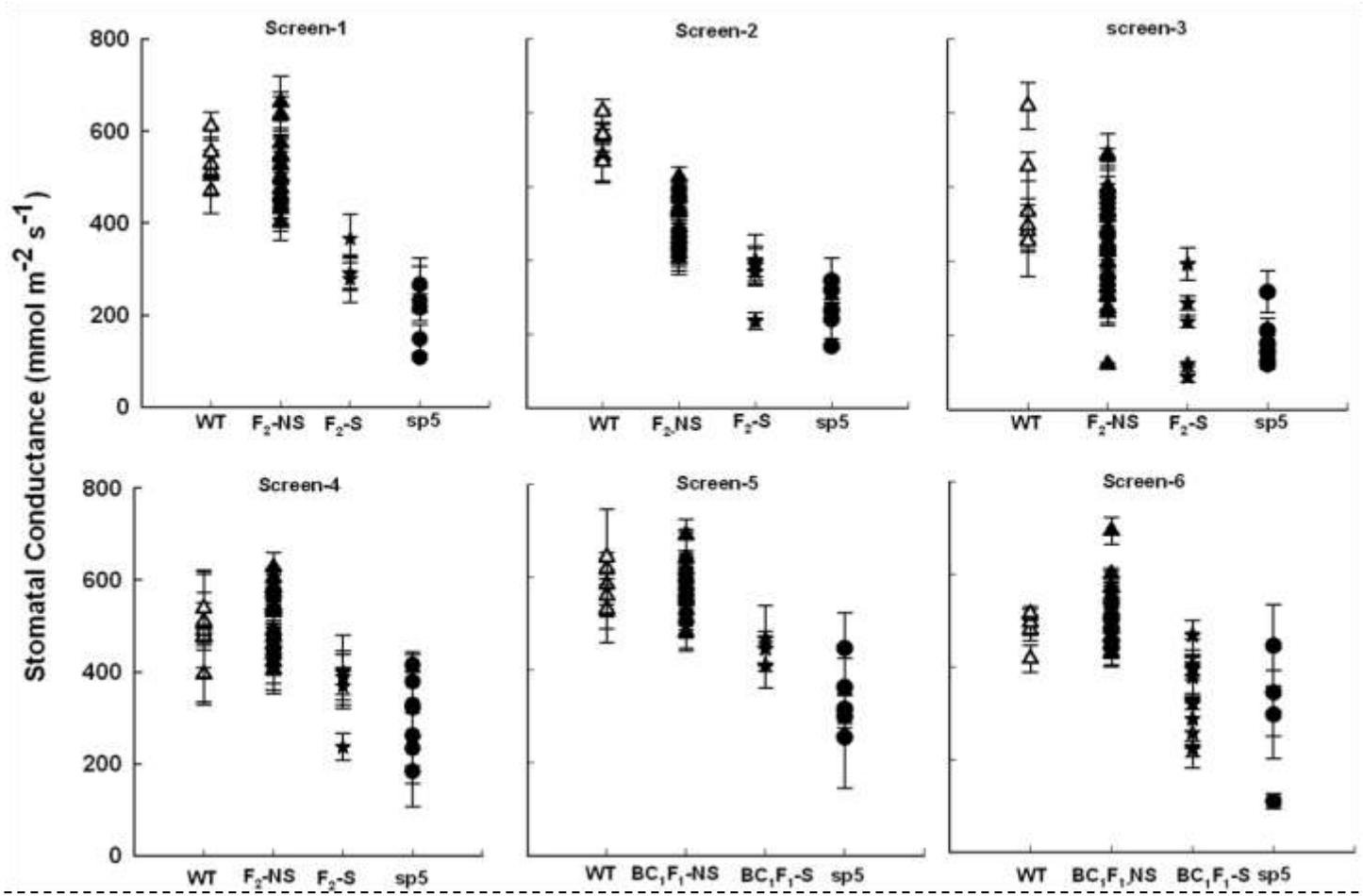

Figure 4.5. Leaf stomatal conductance data collected under controlled environmental conditions. Error bars represent the standard error of the mean for multiple porometer measurements of each individual plant. There were 6 blocks in each screen and each block contained four test plants and one wild type and one sp5 plant as controls. Test plants were either *sAc*×*Ds*-F_2 (screen-1-4) or BC_1F_1 (screen 5&6) generation. NS, not selected for further work; S, selected for further work. Note: All screens were carried out at different .

The g_s Data collected from the screening process (Figure 4.5) showed that the population of *sAc*×Ds-F_2 plants had a greater standard deviation (±129) compared to the control plants (i.e. standard deviation was ±115 and ±113 mmol m^{-2} s^{-1} for wild type and sp5, respectively) (Table 4-2). Mean g_s data collected from these screens was analysed using a T-test assuming unequal variance. This showed that *sAc*×Ds-F_2 plants had statistically lower mean g_s value (455 mmol m^{-2} s^{-1}) than the wild type plants (526 mmol m^{-2} s^{-1}) at ($P < 0.001$). The lowest mean g_s value was recorded in the genotype sp5 (256 mmol m^{-2} s^{-1}), this was significantly lower than both *sAc*×Ds-F_2 and wild type plants ($P < 0.001$).

Table 4-2:Data collected from mean g_s values of genotypes under controlled environmental conditions

Genotype	Mean g_s (mmol m^{-2} s^{-1})	Standard deviation (±)	No. of plants
sAc×*Ds*-F_2	455	129	240
WT (Ailsa Criag Tm2[a])	526	115	60
sp5	256	113	60

Similarly, g_s data obtained from *sAc*×Ds-F_2 plants revealed a more normal distribution (range 180 to 720 mmol m^{-2} s^{-1}) compared to the wild type (range 380 to 975 mmol m^{-2} s^{-1}) which was skewed towards higher values, and sp5 (range 185-645 mmol m^{-2} s^{-1}) which was skewed towards lower values (Figure 4-6).

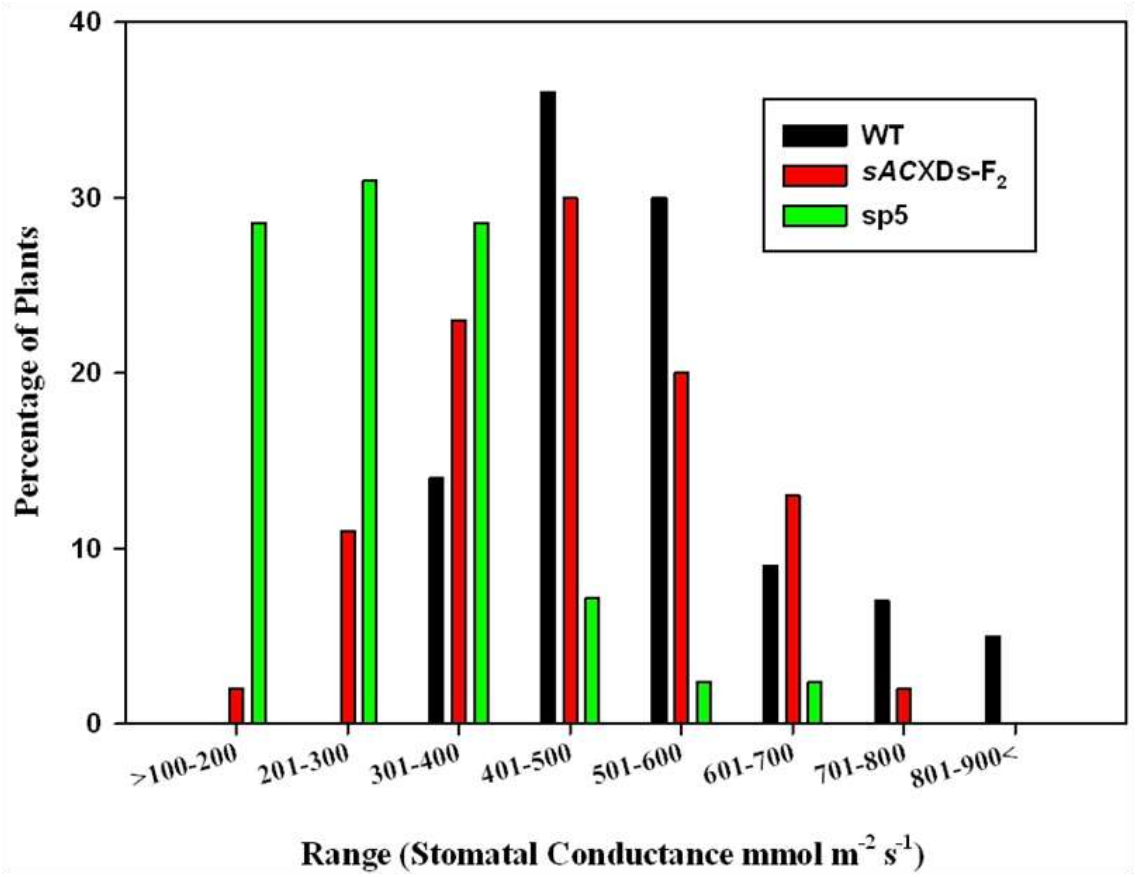

Figure 4-6. Stomatal conductance of wild type and *sAC*×*Ds*-F_2 plants recorded under control environment. Data was pooled from all 9 screens. T-test assuming unequal variance revealed statistically significant difference ($p< 0.001$) between the genotypes.

As a result of the g_s screening process, 41 *sAc*×Ds-F_2 plants were selected primarily on the basis of their low g_s and secondarily interesting phenotype (e.g., rapid growth and leaf morphology). In some cases *sAc*×Ds-F_2 plants had reduced g_s but were not selected for further analysis due to their slow growth and development. A few plants with relatively high g_s but lower than wild type were also selected from BC_1F_1 plants for seed bulking due to their interesting phenotype (e.g., fast growth with high leaf angle from stem, silvery grey leaf colour, and reduced leaf epinasty) similar to phenotypes observed in the high ABA genotype sp5 (Thompson et al., 2007).

4.2.5 Estimating g_s by thermal imaging

Thermal images of 7, 14, 28 and 42 day-old *sAc*×Ds-F_2 plants and control plants (WT and sp5) were collected in the glasshouse and controlled environments using a thermal camera (IVN-770 P, Impac Infrared GmbH, Germany) to assess the leaf temperature of different genotypes as a proxy for transpiration rate (Chaerle et al., 2004). Unfortunately no differences in leaf temperature could be detected between plant genotypes when the thermal images were visually inspected at any time during the growth period.

4.2.6 Stomatal conductance data collection by using IRGA and Porometer

During the initial g_s screening (screen 2 & 3), data was collected from *sAc*×Ds-$F_{2,\ wild\ type}$ and sp5 plants by using both IRGA and Porometer from the same leaf under control environment. The g_s data obtained from both instruments was plotted to look for any correlation. Data revealed that there was a positive linear correlation (r^2 = 0.43, Figure 4-7).

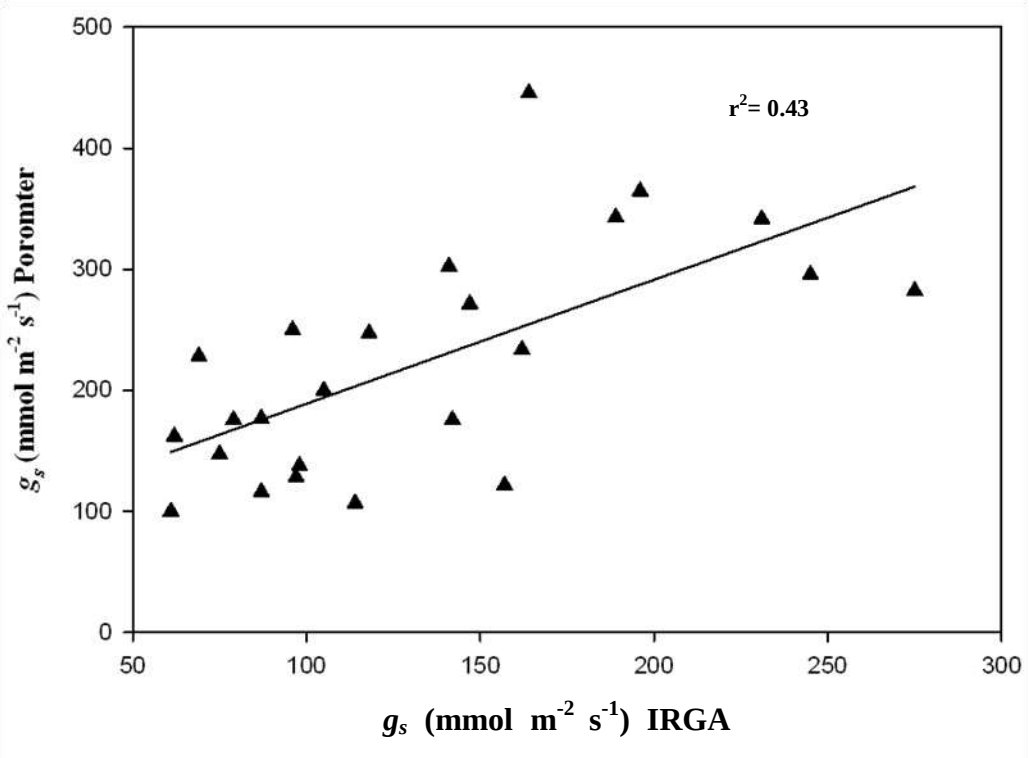

Figure 4-7. Correlation between the g_s data collected from approximately 40-42 days old plants by using Porometer and IRGA (Infrared Gas Analyser) under controlled environmental conditions.

4.2.7 Southern blot analysis to reveal the presence of *Tr-Ds* elements

To ascertain the successful integration of *Tr-Ds* elements into the genome, Southern blots were carried out using genomic DNA prepared from *sAc*×Ds-F_2 plants that had been digested using *Spe*-I and *Nde*-I restriction enzymes. These enzymes were selected because they did not cut inside the NCED gene, hence, making the fragment sizes different for each *Ds* integration event. A probe containing the complete ORF of *LeNCED1* gene was hybridized to the blots. Analysis of the sequence of *LeNCED1* gene, accession AJ439079 revealed that the expected size of the *LeNCED1* band from the endogenous gene on the blot was 3475 bp (Figure 4-8-A). The expected size of the

Ds band when using these two enzymes was 8051 bp for T-DNA-Ds (Figure 4-8-B) and 3419 bp for *Tr-Ds* element (Figure 4-8-C).

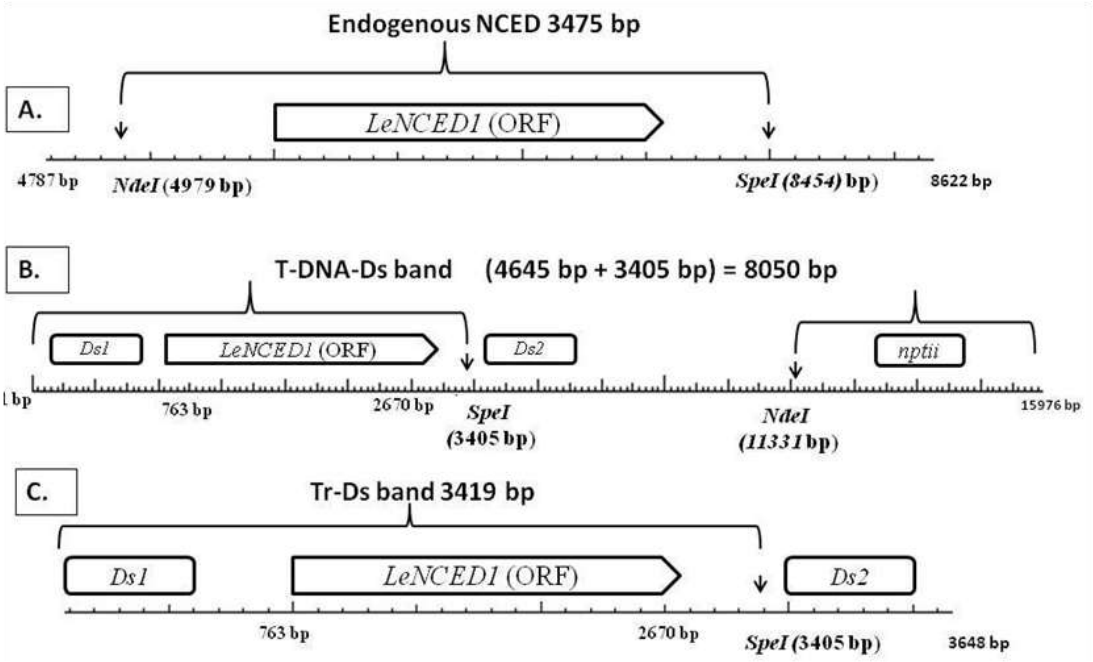

Figure 4-8. The linear sequence of the open reading frame (ORF) from endogenous *LeNCED1*gene (A) the construct pBP6 (B) and transposed *Ds* element in (C) revealing the restriction sites by using *NdeI* and *SpeI* as restriction enzymes. Note the inverted brackets show the expected band sizes by using restriction enzymes *NdeI* and *SpeI* which would hybridize to the NCED probe.

Southern blot analysis showed that there were multiple unique transposition events in the individual *sAc*×Ds-F_2 genotypes (Figure 4-9). Southern data also revealed that the parent line (Ds517-1) transformed with the T-DNA-Ds element had two T-DNA insertions. Genotype sp5 was included in the blot to ascertain that there was no accidental selection of sp5 lines during the screening programme.

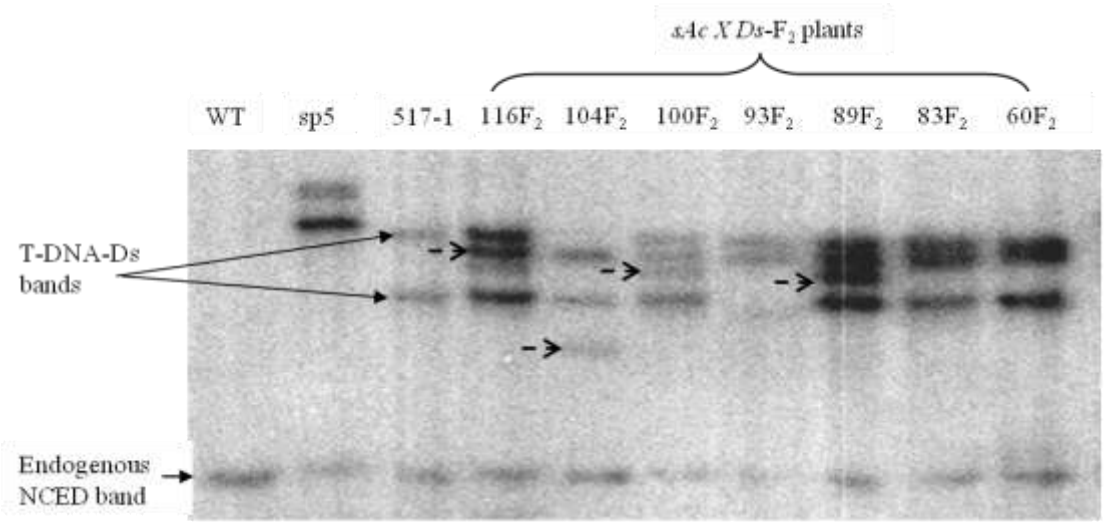

Figure 4-9. A Southern blot analysis to reveal the transposition of *Ds* (Sinclair et al.) elements in selected *sAc*×*Ds*-F_2 plants. Where wild type (WT), sp5 and 517-1 (parent line transformed with the T-DNA-Ds element) lines were used as control. Dashed arrows represent the transposed Ds elements in *sAc* × *Ds*-F_2 plants.

4.3 Discussion

4.3.1 A large variation in plant phenotype and g_s in *sAc*×Ds-F_2 plants

The *sAc*×Ds-F_2 genotypes potentially over-expressing *LeNCED1* at different levels in the genome had varied phenotypes. A large variation in the rate of seed germination was observed. Most of the *sAc*×Ds-F_2 seeds germinated on water but some could not germinate even on D4 or norflurazon, inhibitors of ABA biosynthesis (Bartels and Watson, 1978). These results were in agreement with published reports (Thompson et al., 2000, Qin and Zeevaart, 2002, Hu et al., 2010) showing that NCED1 overexpression delayed the seed germination due to over accumulation of ABA in the germinating embryos.

While the presence of the *Ds* element(s) delayed the seed germination, it was also observed visually that there was a large variation in the rate of plant establishment. This might be attributed to varying levels of *LeNCED1* expression in these plants. Some s*Ac*×Ds-F_2 plants grew at the same rate as wild type and others at a similar rate to the slower sp5 (visual observations only) which establishes at a very slow rate (Thompson et al., 2007, Hwang et al., 2010). Similar observations were made by Hwang et al. (2010) when they overexpressed rice NCED3 gene in *A.thaliana,* which resulted in increased seed dormancy and smaller round leaves. These early observations of germination and establishment, suggested that the transposition of the *Ds* element might have been responsible for creating variation in NCED expression, although it is not possible to attribute the effects to genotype rather than environment without further well-designed experiments.

4.3.2 High throughput plant screening on the basis of g_s

One of the main objectives of the study was to screen plants transpiring at different rates by using robust techniques so that a large number of plants could be studied during this time span. For this purpose, various approaches were adapted including the use IRGA, Porometer and thermal imaging analysis (Jones, 1999, Takai et al., 2010) . It was observed that the thermal imaging technique could not be successfully used to screen plants rapidly either under the glasshouse or in the controlled environment cabinet set at 22^{o}C and 65 % relative humidity. Increasing the temperature (Deswarte, 2007) above 22^{o}C or reducing the humidity below 65% could be an option in order to observe any surface temperature differences between genotypes, because at

lower humidity and higher temperatures the rate of transpiration would tend to be generally higher (Collatz et al., 1991).

4.3.3 The Choice of equipment: Porometer or IRGA (Infra red gas analyser)?

More plants were screened per unit time by using the Porometer as compared to IRGA, because the Porometer was easier to use and multiple readings from each plant could be collected in a short period of time saving energy, cost and time without compromising on the quality of data obtained (e.g. Figure 4-7 where the Porometer was able to distinguish between the g_s from wild type and sp5 plants with a known difference observed during the previous studies at Warwick, HRI).

Several environmental factors influence g_s, including light (Bunce, 2000), vapour pressure deficit (VPD) (Day, 2000), and CO_2 concentration gradient between leaf and its microclimate (Robredo et al., 2007, Bunce, 1998). Initially *sAc*×Ds-F_2 plants were screened for g_s under natural light conditions in the glasshouse, but it was observed that during light fluctuation (sunny and cloudy) periods there was a dramatic change in the g_s of the same genotype. Hence the genotypes were screened under control environmental conditions to get more consistent data.

Leaf g_s is also controlled by the concentration of ABA in the xylem sap. A higher ABA concentration in the xylem sap reduces the leaf g_s (Tardieu et al., 1992, Zhang and Outlaw, 2001, Thompson et al., 2007). The *sAc*×Ds-F_2 genotypes used in this study exhibited a large variation in g_s which might be attributed to the variation in the expression of *LeNCED1*. This variation included a few *sAc*×Ds-F_2 plants with much

higher g_s than wild type plants. This might be attributed to the co-suppression of endogenous *LeNCED1* expression (Thompson et al., 2000, Smith et al., 1990).

4.3.4 Tightly linked *Ds element*s in *sAc*×-F_2 plants

The exact pattern of inheritance of mutation (needle like leaves) in plant phenotype (described in section 4.2.2) could not be calculated because the seedlings containing the *sAc* element, potentially inheriting this phenotype, were discarded and this phenotype could only be identified in approximately 14-21 days old plants. This might be due to integration of the *Ds* element and disruption of the function of gene(s) (Martienssen, 1998) contributing towards the leaf expansion and development.

Southern blot data in Figure 4.9 showed that there were two copies of the T-DNA-Ds element present in the Ds517-1 line that was the donor parent for the T-DNA-Ds element. Both copies were present in all tested F_2 genotypes except $104F_2$, where only one copy of the T-DNA-Ds element was present. This suggests that although the two T-DNA-Ds elements were genetically linked since they predominantly occurred together, they had been separated in one line. In total 115, *sAc*×Ds-F_2 and *sAc*×Ds-F_3 lines were tested through Southern blotting and one recombinant was found this shows that the distance between the two insertions was only 0.86 cM. (No. of recombinants/total population × 100=0.86).

Alternatively, it can also be postulated that although the transposition of the *Tr-Ds* element was mainly by a replicative mechanism (Feschotte and Pritham, 2007). In case of $104F_2$, the transposition might have occurred by a cut and paste mechanism, and this may have been responsible for the loss of one of the original T-DNA bands, rather than by a recombination event.

Data obtained from Southern blot analysis of *sAc*×Ds-F_2 plants (Figure 4.9) and *sAc*×Ds-F_3 progeny (Chapter-5) demonstrated that there were multiple transposition events in these plants which might explain a slightly skewed segregation ratio of *Ds* containing GUS negative plants from number of GUS negative *Ds* containing expected. However, this was not a significant skew at the 5% probability level (P = 0.08). Presumably the ratio did not alter significantly because most of these transposition events were local and tightly linked to the T-DNA-Ds.

Several unique transposition events in *sAc*×Ds-F_2 genotypes might have contributed towards lower g_s and distinct plant morphology depending on the position of *Tr-Ds* element. Altmann et al. (1995) used *Ac/Ds* transposition system in their study and found unique plant morphology in *A.thaliana* on transposition of *Ds element*s to unique sites in the genome (Altmann et al., 1995). If the *Ds* element transposed near an enhancer region then the level of expression of *LeNCED1* would have increased; wheras a transposition event near a silencer region would result in decreased *LeNCED1* expression. It is also possible that a *Tr-Ds* would have occurred in a position that would allow aberrant antisense transcripts to be produced, resulting in co-suppression of the endogenous gene (PalBhadra et al., 1997) and also in the high g_s and possibly increased epinasty observed in some *sAc*×Ds-F_2 plants.

4.4 Concluding remarks

The result of the screening programme of *sAc*×Ds-F_2 plants revealed a large variation in plant phenotype including seed germination and g_s compared to wild type plants. Southern blot analysis also revealed transposition of *Tr-Ds* element in different *sAc*×Ds-F_2 lines indicating a successful use of *sAc*/*Ds* strategy in tomato plants. The

selected genotypes were further analysed for the unique transposition events in the genome and their respective role in plant g_s. This has been described in the next chapter.

Chapter-5 Initial genetic and phenotypic assessment of selected genotypes

5.1 Introduction

In Chapter-4, *sAc*×*Ds*-F_2 plants containing the *Ds* element were screened in the controlled environment for reduced g_s and genotyped using PCR and Southern blotting to reveal any transposition events. In the current chapter, progeny from previously selected genotypes i.e. $116F_2$, $102F_2$ and $59F_2$ were investigated for any unique and stable transposition event(s) of *Tr-Ds* element and their effect on seed germination and g_s.

5.2 Materials and methods

5.2.1 Plant material available for g_s screening in F_3 population

The F_2 plants selected during g_s screening were allowed to self pollinate in the glasshouse. Only 17 F_2 plants produced enough F_3 seed by self-pollination for further studies; the remaining lines had poor seed set due to boxy fruits. Three F_3 families namely $116F_3$, $102F_3$ and $59F_3$ were screened for their g_s under controlled environmental conditions.

5.2.2 Measurement of g_s and statistical analysis of data

Approximately 40 days after germination (DAG), plants were grouped in eight randomized blocks. Each block contained six F_3 plants and one from each control line (wild type and sp5). These plants were randomized in each block and data was collected four times during a day using a hand-held SC-1 porometer (Decagon devices Inc. USA). If the g_s readings obtained were relatively consistent and standard deviation was

acceptable, only four porometer measurements were considered to be enough, otherwise plants were left in the Weiss room overnight for further g_s data collection on a second day. Stomatal conductance data was analysed using Genstat v.12 (VSN, International Ltd. Oxford, UK) to perform an Analysis of Variance (ANOVA), and differences were calculated by the comparison of means.

Chi-square test was used to measure 'goodness to fit' genotypic ratios in F_3 and F_4 population by using the following formula (Zar, 1989);

$$\chi^2 = \sum_{i=1}^{k} \frac{(f_i - f_e)^2}{f_e}$$

Where 'x' is the value of chi from chi square distribution table, 'k' represents the number of genotypic categories, f_i is the number of counts observed in class 'i' and 'f_e' is the expected number of counts in class 'i'.

5.3 Results

5.3.1 Genotype 116F_3

The parent plant (116F_2) germinated on 1.0 mM D4. This plant had a unique phenotype with shorter petioles and more leaves per unit length of stem when compared to wild type plants (visual observation only). This parent plant also had almost 50% lower g_s compared to the wild type plants (Table 5-1), but the values were 16% higher than sp5 plants.

Table 5-1: Leaf stomatal conductance (mmol m^{-2} s^{-1}) of $116F_2$ in comparison to the wild type and sp5 genotypes

GT	No. of plants	Mean g_s	*SEM	[1]p-value	Statistical significance
****116F$_2$**	1	260.8	35.1	n/a	n/a
wild type	6	573.5	67.2	0.003	yes
***Sp5**	6	185.3	24.7		

* represent standard error of means, calculated from multiple g_s values obtained from each plant.
**Genotype $116F_2$ germinated on 1.0 mM D4
***sp5 germinated on 3.3 μM norflurazon
[1] p-values obtained from t-test assuming equal variance

Note: SEM for $116F_2$ is for variation between readings on one plant, and SEM for wild type and sp5 is for plant to plant variation.

In total, 120 seeds of genotype $116F_3$ were incubated on water in Petri dishes for three days. Seeds that failed to germinate after three days were transferred to 3.3 μM norflurazon, in an attempt to stimulate further germination (Thompson et al., 2000). Only 7.5% of the seeds germinated three days after imbibition (DAI) on water, whereas, 82.9% seeds had germinated after a further two days on norflurazon.

5.3.1.1 Genomic analysis of $116F_3$ by Southern blot

Genomic DNA isolated from a population of $116F_3$ plants (accession AT16780) was analysed by Southern hybridization to identify the presence and segregation of specific *Tr-Ds* and T-DNA-Ds bands. The probe contained a complete ORF of *LeNCED1* gene and so would detect both the endogenous *LeNCED1* gene, and any transgene or *Tr-Ds* containing this sequence. Southern blots revealed three independent insertions of the *Tr-Ds* element (Figure 5.1-A &B) in the genome of $116F_3$ plants. These were named *Tr-Ds*-1, *Tr-Ds*-2 and *Tr-Ds*-3.

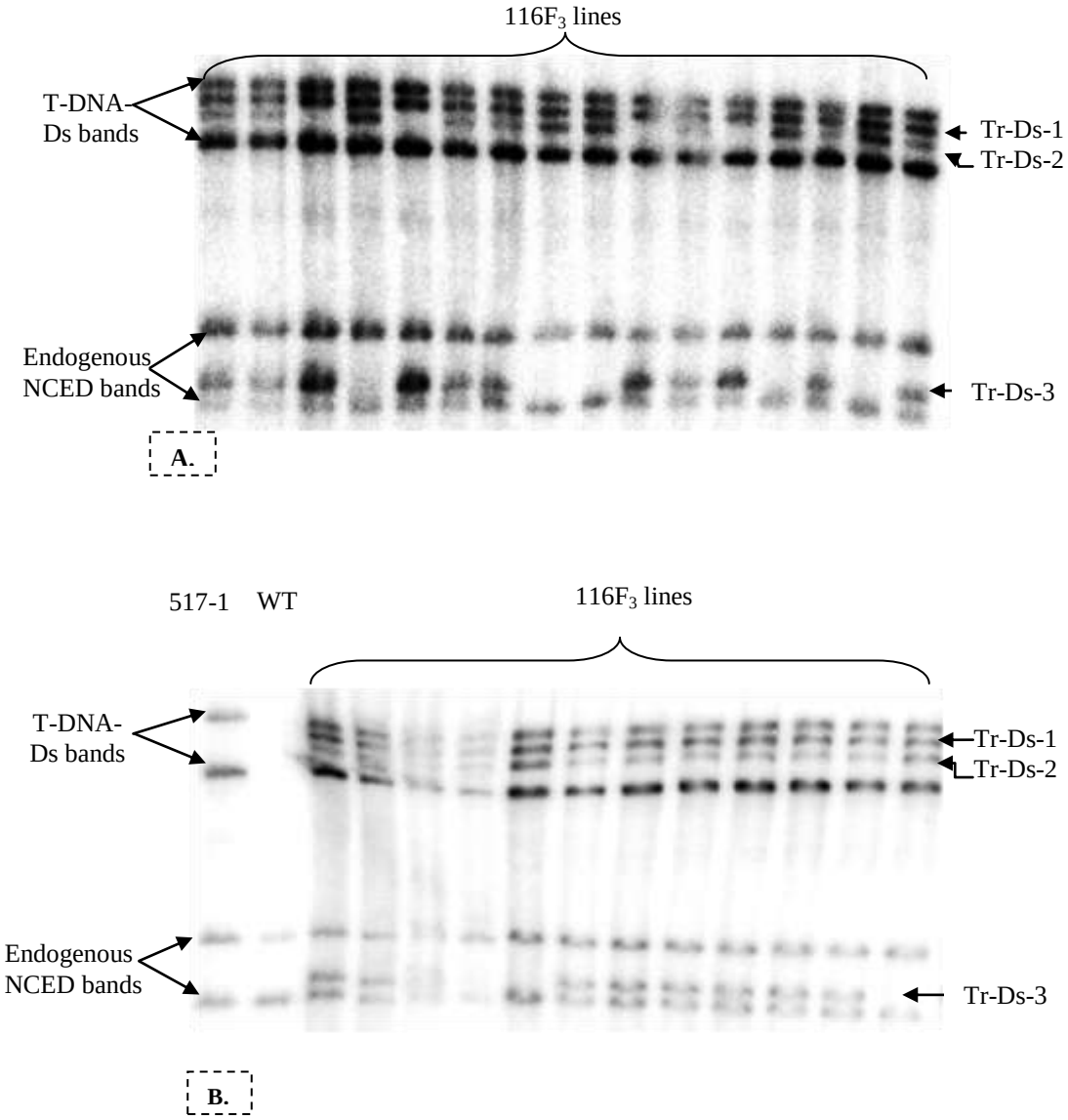

Figure 5.1. Southern blot analysis of transposition events in genotype $116F_3$. The blots were probed with the *LeNCED1* gene. Each track in the group labelled $116F_3$ lines represents an individual $116F_3$ plant; Tr-Ds-1, Tr-Ds-2 and Tr-Ds-3, bands arising from transposition; T-DNA-Ds, the original T-DNA-Ds from line 517-1; the two "Endogenous NCED bands" represent *LeNCED1* and a related gene. In panel **B,** the 517-1 line and wild type were

Locus *Tr-Ds*-1 and the two T-DNA-Ds loci were present in all 49 plants from $116F_3$ analysed through Southern blotting, consistent with homozygosity for all three bands. Both *Tr-Ds*-2 and *Tr-Ds*-3 elements segregated consistent with a 3:1 ratio, as

tested by Chi-squared test (Table 5-2), suggesting that each locus was heterozygous in the $116F_2$ parent plant.

5.3.1.2 Linkage analysis for *Tr-Ds*-2 and *Tr-Ds*-3:

Further analysis was carried out on the segregation ratios of the *Tr-Ds*-2 and *Tr-Ds*-3 loci to find out whether they were genetically linked. Data (Table 5-2) suggested that the parent plant ($116F_2$) was heterozygous for both *Tr-Ds*-2 and *Tr-Ds*-3 loci. The possible outcomes from self-pollination of the heterozygous $116F_2$ plant (genotype *Tr-Ds*-2.+/*Tr-Ds*-3.+) in the F_3 generation, and a linkage analysis are given in Table 5-3**.**

Table 5-2: Number and segregation ratio of $116F_3$ lines classified according to the presence of *Tr-Ds* loci in each plant

GENOTYPE	Bands Present	No.of plants with/without band	Ratio	$x^2_{cal.}$	$x^2_{0.05,1}$	Ratio 3:1
$116F_3$	*Tr-Ds*-1	49/0	n/a	n/a	n/a	n/a
$116F_3$	*Tr-Ds*-2	41/8	5.1:1	1.967	3.841	Yes
$116F_3$	*Tr-Ds*-3	36/13	2.8:1	0.081	3.841	Yes

Where $x^2_{cal.}$ indicates the calculated Chi Square value

$x^2_{0.05,1}$ shows the value from Chi Squared table at p value 0.05 at 1 degree of freedom

The presence of each of the *Tr-Ds* elements, as detected by Southern blot, is indicated as a 2 or 3. Assuming that *Tr-Ds*-2 and *Tr-Ds*-3 loci were linked, the gametes indicated in italics were recombinant and those underlined were non-recombinant.

The phenotypic ratio for the dihybrid cross of two dominant loci assuming independent segregation (presence or absence of the two *Tr-Ds* bands) is 9:3:3:1 for the 49 plants assayed in the Southern blot was 9.1: 3.0:3.9:0. (Table-5-3) which was not different to the Mendelian 9:3:3:1 ratio (χ^2 = 3.93 compared to 7.82 as the χ^2 value for $P < 0.05$ with three degrees of freedom). Therefore *Tr-Ds*-2 and *Tr-Ds*-3 loci appeared to be segregating independently.

Table 5-3: Segregation ratio of Transposed *Ds element*s in genotype 116F$_3$

Gametes	No. of plants expected	No. of plants observed	$x^2_{calc.}$	$x^2_{0.05,3}$	Ratio 9:3:3:1
Tr-Ds-2.*Tr-Ds*-3	27.6	28	3.93	7.82	Yes
Tr-Ds-2.+	9.19	9			
+.*Tr-Ds-3*	9.19	12			
++.++	3.06	0			
Total	49	49			

Where x^2_{cal}.was the calculated Chi Square value. $x^2_{0.05,3}$ was the value from Chi Square table at *p*,0.05 and 3 degrees of freedom.

5.3.1.3 Stomatal Conductance of genotype 116F$_3$

Data collected for g_s indicated that the 116F$_3$ family had almost 20% lower g_s than wild type, and this was significant at $P < 0.01$. This genotype on average had 16% higher stomatal conductance than sp5 plants ($P < 0.01$; Fig 5.1). To confirm that the low stomatal conductance was caused by the presence of *Ds element*(s), plant genomic DNA was extracted and PCR was carried out to confirm the presence of the *Ds* element by using the primers *Ds1For2* and *notRev5* (Appendix-I). Results of PCR data for the *Ds* element from 74 plants revealed that all plants genotyped contained the *Ds* element, as expected from the Southern blot results which showed that *Tr-Ds*-1 was homozygous

and should therefore be present in all F_3 plants, whereas, *Tr-Ds*-2 and *Tr-Ds*-3 were segregating as a single copy with dominant expression.

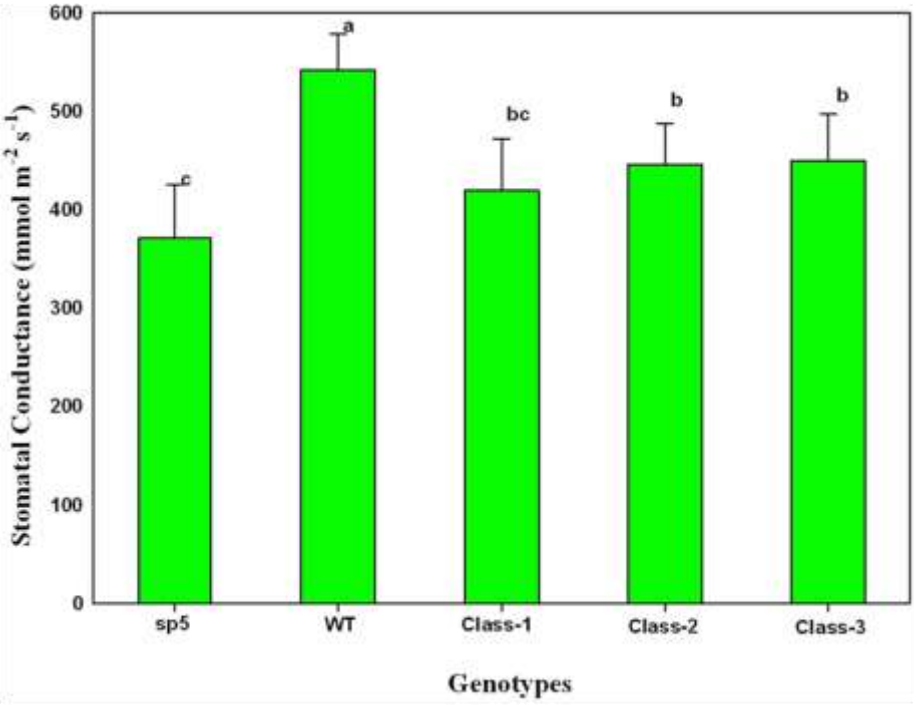

Figure 5.2. Stomatal conductance of $116F_3$ recorded under control environment conditions. Classes 1-3 represent g_s data of plants with different banding pattern observed through Southern hybridization (for details see Figure 5-1 A&B and text). Different letters indicate statistically different means ($P < 0.01$). Error bars represent the standard error of means.

To find out which particular locus was responsible for low g_s in $116F_3$ lines, g_s data were analysed as three separate classes. Class-1 contained all three *Tr-Ds* loci, class-2 contained *Tr-Ds*-1 and *Tr-Ds*-2, and class-3 contained *Tr-Ds*-1 and *Tr-Ds*-3 loci. The mean g_s from each of the three classes was calculated and compared by T-test (two samples assuming equal variance). Data revealed that statistically there was no significant difference between the three classes (Figure 5-2). It therefore appears that the low g_s might be attributed to the *Tr-Ds*-1 band or the T-DNA-Ds bands as they were

present in all the 116F_3 plants. Data are consistent with the possibility that the remaining two loci did not contribute in the reduction of leaf g_s because the presence of *Tr-Ds-2* and or *Tr-Ds-3* bands in the segregating population did not affect the plant g_s.

5.3.2 Genotype 102F_3

5.3.2.1 Germination of 102F_3 and discovery of a novel root mutation

The 102F_2 parent plant had very mild symptoms of interveinal flooding with relatively slower growth and establishment (visual observation) compared to the wild type. It also had lower g_s (Table 5-4) compared to wild type plants and this was the basis of selection in chapter 3. The F_3 seeds (accession AT1675) were germinated on water only. It was noted that up to 100% seed germination was achieved within 72 hours of imbibition, faster than the wild type. Soon after the seed germination, the radicle elongation slowed down in 25.7% of the seedlings (19/74) with a brown tissue appearing at the terminal end of the radicle.

Figure 5.3. Root mutation in genotype 102F_3. Left, wild type seedling; right, 102F_3 seedling, note a brown terminal end of the radicle and emergence of lateral roots behind this brown tissue.

Table 5-4: Leaf stomatal conductance (mmol m^{-2} s^{-1}) of $102F_2$ in comparison to the wild type and sp5 genotypes

GT	No. of plants	Mean g_s	*SEM	[1]p-value
**$102F_2$	1	402.4	45.11	n/a
wild type	6	729.2	47.8	<0.01
***sp5	6	291.8	43.5	

* represent standard error of means, calculated from multiple g_s values obtained from each plant.
**Genotype $102F_2$ germinated on water alone
***sp5 germinated on 3.28 μM Norflurazon
[1] p values obtained from statistical analysis using t-test assuming equal variance

5.3.2.2 Signs of interveinal flooding

The germinated seeds from $102F_3$ were sown in the compost. Later on (6-7 days after germination) it was observed that almost 31% of the seedlings (21 out of 68) failed to expand their cotyledons and formed a "lollipop" shape with testa still enveloping the cotyledons. In such instances the testa was removed by using a pair of fine forceps, failure to do this resulted in seedlings death at this stage. Similar observation was made by Tung et al. (2008), while working with tomato plants over-expressing *LeNCED1* to a high level (Tung et al., 2008).

It was also observed that at relatively high temperature (20-22^oC) accompanied by high humidity (≥ 65%) the interveinal flooding increased in $102F_3$ genotype, this phenomenon was especially evident when these plants were kept in controlled environment at a constant humidity of 65% overnight (Figure 5-4-B&C). Further, it was noticed that the plants guttated in the early hours of the morning (Figure 5-4-C). Similar observations had previously been made in plants over expressing *LeNCED1* gene with strong promoters like rbcs and the "Gelvin "super promoter", but at much higher relative humidity (95%) (Thompson et al., 2000, Tung et al., 2008).

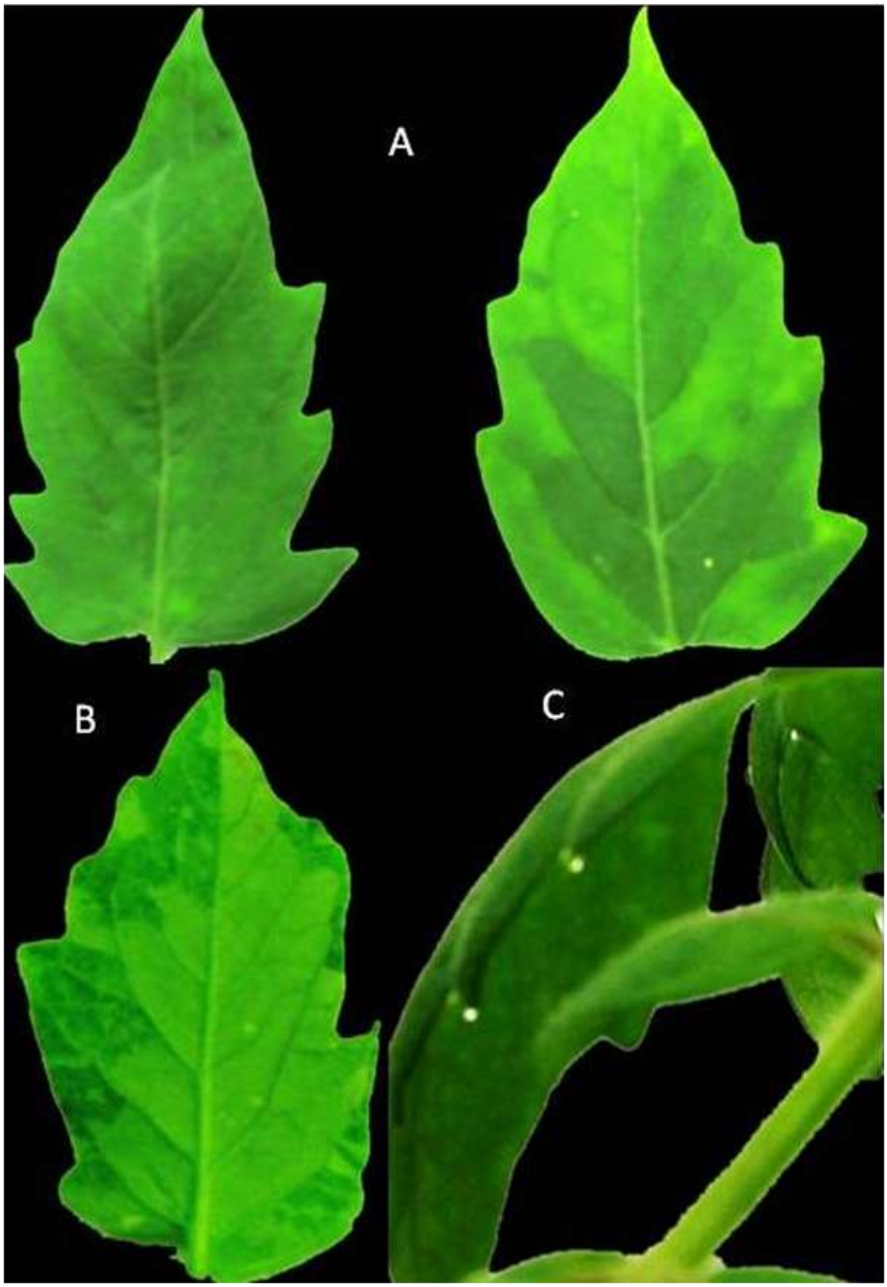

Figure 5.4. Leaf phenotypes in genotype $102F_3$. **A**. interveinal flooding observed from the adaxial side of leaf from plants, wild type on the left $102F_3$-97 on the right. **B**. abaxial side of leaf from genotype $102F_3$-97 **C.** Plants ($102F_3$) kept under 65% constant humidity overnight in the Weiss room showed guttation from the leaf edges.

5.3.2.3 Southern blot to reveal transposition events

Southern hybridization (Figure 5-5) demonstrated that there were two unique transposition events in this genotype. Both loci, namely *Tr-Ds*-4 and *Tr-Ds*-5, were genetically linked to each other and to the T-DNA-Ds elements. In total, 32 plants were analysed by Southern blotting, and 25 plants (78%) had both transposed and the two T-DNA-Ds elements, wheras the remaining 7 plants (22%) had lost all T-DNA-Ds and *Tr-Ds* elements. No plants were observed that had only T-DNA-Ds or only *Tr-Ds* element. This suggested that all the transposed and non transposed elements were linked. The linked complex of *Ds* sequences segregated as a single locus in a 3:1 ratio confirmed by Chi-Square test (Table 5-5).

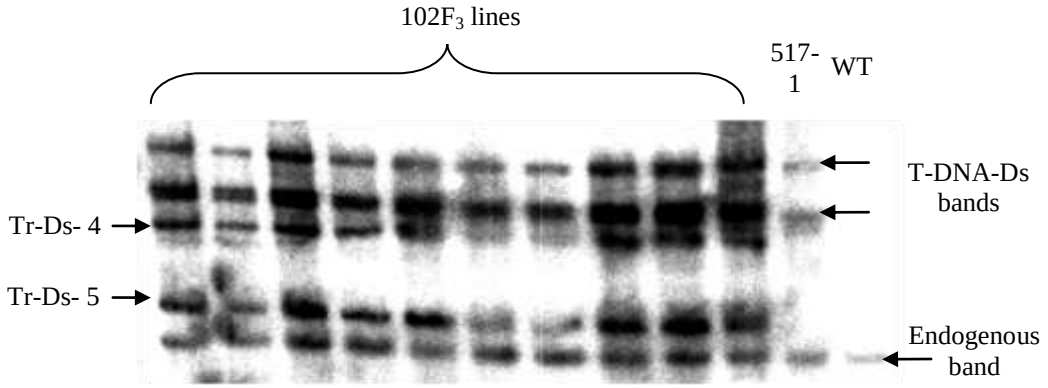

Figure 5.5. A Southern blot analysis revealed that two transposed *Ds* (Sinclair et al.) bands were present in the $102F_3$ family (self pollination of $102F_2$ line). Blot was hybridised with the probe containing *LeNCED1* ORF. 517-1 line and wild type were used as control plants.

Table 5-5: Calculation of segregation ratio of *Ds element* in genotype $102F_3$ by using Chi square test.

GT	Bands Present	Number of plants observed	Number of plants expected	$x^2_{calc.}$	$x^2_{0.05,1}$	Ratio 3:1
$102F_3$-Ds^+	T-DNA-Ds + Tr-Ds + endogenous	25	24	0.167	3.841	Yes
$102F_3$-Ds^-	Endogenous only	7	8			
Total		***32***	***32***			

Where x^2_{cal}.was the calculated Chi Square value

$x^2_{0.05,1}$ was the value from Chi Square table at p,0.05 and 1 degrees of freedom.

In addition to Southern blot analysis, PCR was carried out to determine segregation ratio of T-DNA-Ds and *Tr-Ds* elements. Results showed that the presence of *Ds* element was segregating 3:1, 75% of the plants (65 out of 86) were found positive for the *Ds* element. This again indicated that the parent F_2 plant was heterozygous for the *Ds* elements occurring at a single complex locus. During the PCR screen, a duplex PCR was used. Primers *(phytoene synth-F* and *phytoene synth-R*) for the endogenous *phytoene synthase* gene were used to avoid false negative assays (Figure 5-6). Primer pair *Ds1For2* and *notRev5* amplified a band of size 839bp when a *Ds* element was present. But the primers used as internal control can amplify a band of 120bp in all genotypes as the enzyme *Phytoene synthase* is present in all tomato genotypes. The absence of this 120bp band on the gel indicated the failure of PCR reaction.

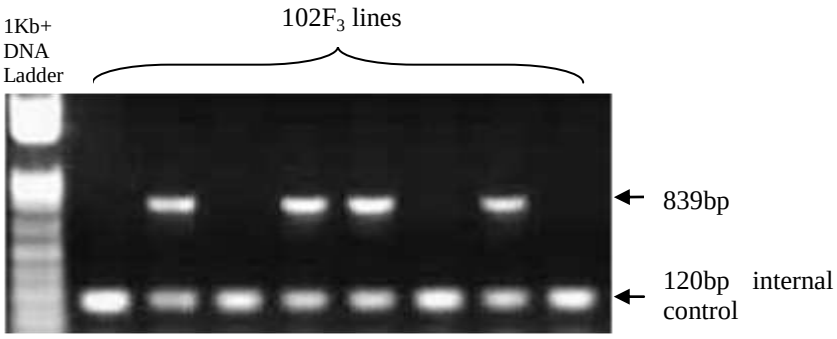

Figure 5.6. A gel image from duplex Ds-PCR of $102F_3$ lines showing internal control. 839bp band shows the *Ds* element whereas 120 bp band was internal control from *Phytoene synthase*

5.3.2.4 Leaf Stomatal Conductance of $102F_3$ lines

Data collected for the g_s from $102F_3$, wild type and sp5 plants under controlled environmental conditions was analysed using ANOVA: $102F_3$ plants containing the *Tr-Ds* element had significantly reduced stomatal conductance compared to the $102F_3$*Ds*negative plants, the wild type and the sp5 plants ($P < 0.01$). The g_s was almost 50% less than wild type and almost 25% lower than the sp5 (Fig 5-7).

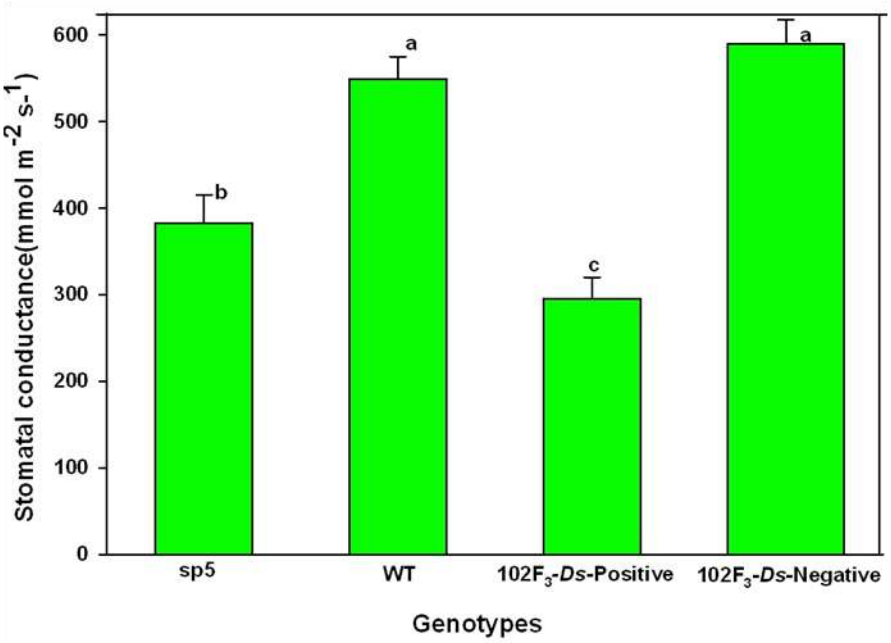

Figure 5.7. Stomatal conductance of genotype $102F_3$ measured under controlled environmental conditions. Letters a, b, and c represent the statistical significance among different lines; the same letter indicate no statistical difference in the g_s. Error bars represent the standard error of means.

5.3.2.5 Viviparous nature of progeny

The seeds in one of $102F_3$ progenies ($102\text{-}97F_4$) germinated inside the fruit which indicated that this genotype might have been viviparous. Further seed germination experiments were conducted on this genotype and the results revealed that some of the seeds started to germinate approximately 18 hours after imbibition (HAI) at 25^oC in the dark. However, this was not true in all of the F_4 lines obtained from self pollination of $102F_3$ plants (Figure 5-8) suggesting that the gene mutation which might be responsible for the precocious seed germination was not homozygous and that it was segregating in the progeny.

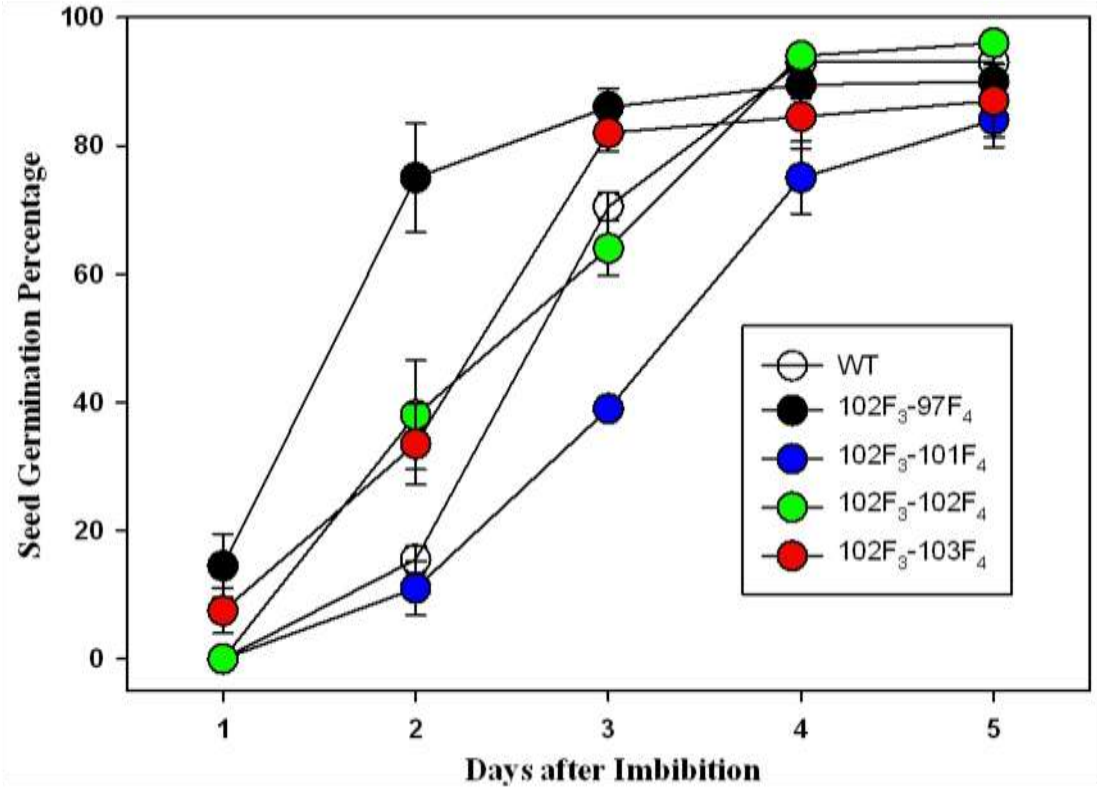

Figure 5.8. Variability in the rate of seed germination of $102F_4$ lines. Fifteen (15) seeds from each genotype were germinated on moist filter papers with two replications. Seeds were observed for the radicle emergence ever 24 hours. Error bars prepresent the range.

5.3.2.6 Effect of exogenous application of ABA on viviparous seed

It was hypothesized that the *Tr-Ds* element might have caused a mutation in the genes involved in germination. For example, a mutation in *ABI3* or *ABI5* would cause the seed to germinate precociously (Lopez-Molina et al., 2002) or may be insensitive to high levels of endogenous ABA in the seed. To test this hypothesis, seeds were grown in the dark at 25^{o}C on minimal agar medium (0.8% agar only) with 10 and 20 μM ABA using wild type seeds as control (Figure 5-9). According to the hypothesis that the seeds were insensitive to ABA, seeds of genotype $102F_3$-$97F_4$ should have germinated faster than wild type on the above-mentioned ABA concentrations. However, the results

showed that germination of genotype $102F_3$-$97F_4$ was more sensitive to ABA in comparison to wild type at 10 and 20μM exogenous application of ABA (Figure 5-9).

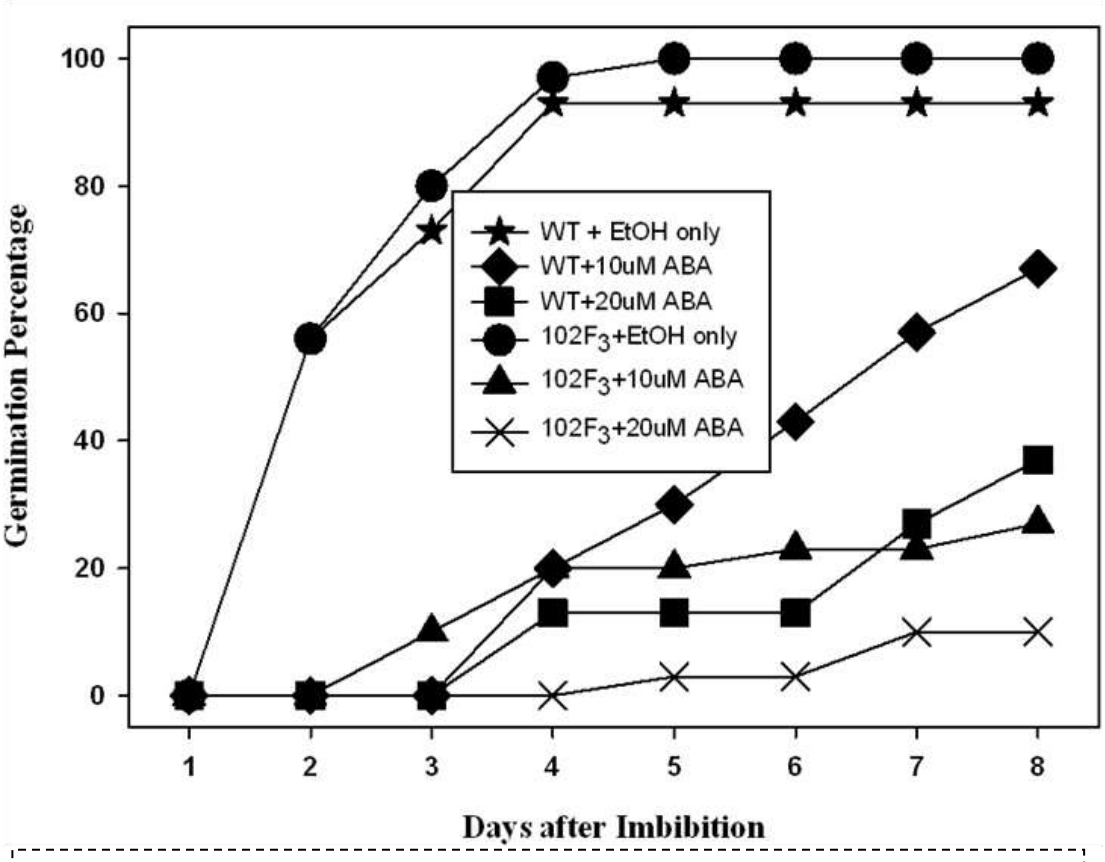

Figure 5.9. Effect of exogenous ABA application on seed germination of $102F_3$-$97F_4$. $102F_3$ and wild type seeds germinated on agar medium containing only 0.8% agar under dark conditions and 25°C. ABA stock solution was dissolved in 97% ethanol (EtOH) at 0.1M. Equal concentration of EtOH was present in each treatment.

5.3.2.7 Production of true breeding progeny of genotype $102F_3$:

The F_4 families were obtained by self pollination of randomly selected F_3 lines containing the *Tr-Ds* element. To obtain a homozygous line, the *Ds* copy number was quantified in one F_4 family derived from a $102F_3$ plant ($101F_4$) that was suspected of

being heterozygous for the single complex *Ds* locus. Copy number was determined using quantitative PCR (Fig 5-10).

The expectation was that the F_4 family should segregate 1:2:1, where 25% population should be homozygous and azygous each and 50% population should have been heterozygous as all the *Tr-Ds* and T-DNA-Ds elements were linked. Data presented in Figure 5-10 could be classified into three distinct quantitative classes and was analysed using chi-square test using 2 d.f. The results showed that there was a 90% probability that the difference between the expected ratio of 1:2:1 and the observed results could be explained by chance alone. The *Ds*-negative control plants (wild type, $101F_3$-$3F_4$ and $101F_3$-$4F_4$) had only two *LeNCED1* copies per diploid genome, representing the homozygous endogenous genes only, in agreement with the Southern blot and PCR data that indicated absence of *Tr-Ds* element. The heterozygous parent plant $101F_3$ contained six copies of *LeNCED1*, but $101F_3$-$26F_4$ and $101F_3$-$31F_4$ contained double this number of copies. This indicated that these two latter plants were homozygous for the *Ds* locus. The homozygous plants selected through q-PCR were grown in the glasshouse and self-pollinated to obtain true breeding F_5 progeny.

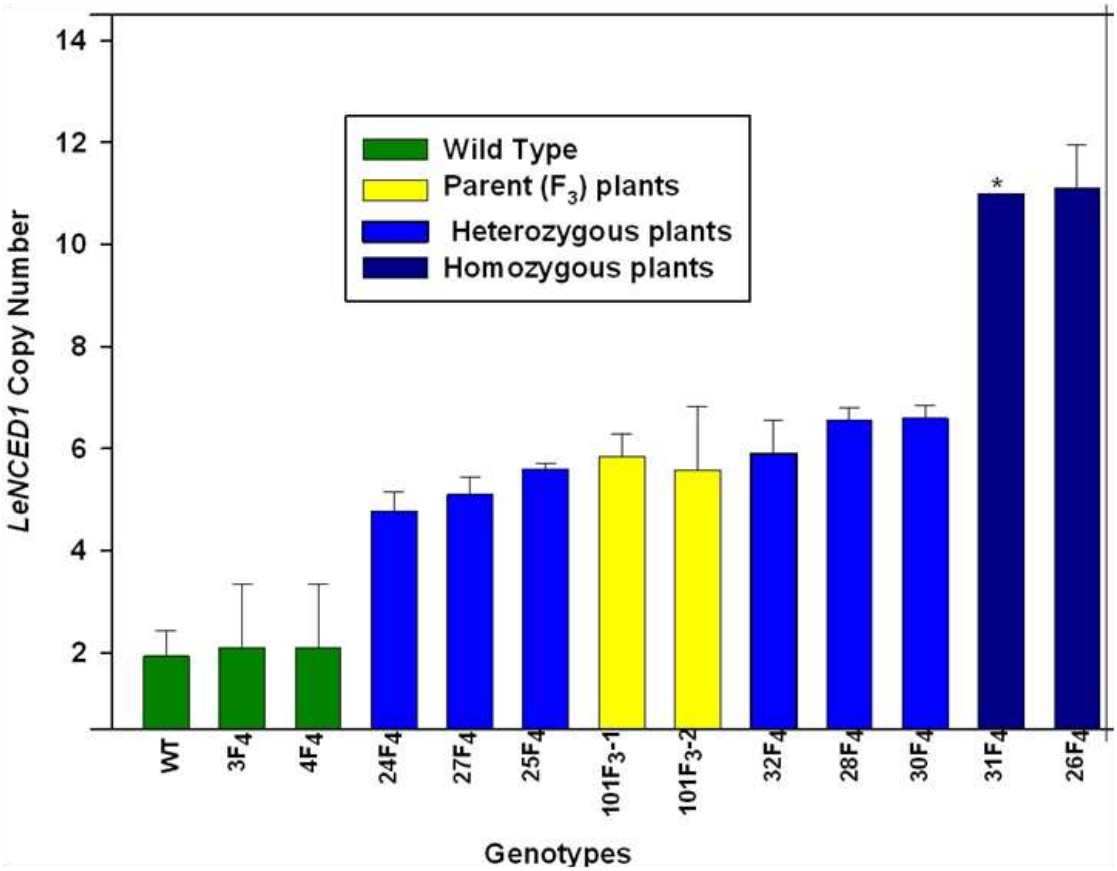

Figure 5.10. Quantitative-PCR data obtained from the $102F_2$-$101F_3$ family derived from selfing of $102F_2$ plant number $101F_3$. There were three biological and two technical repeats in each reaction. $101F_3$-1 and $101F_3$-2 were two separate DNA samples from the same parent plant ($101F_3$), used as heterozygous control and all F_4 plants were its progeny. wild type, $3F_4$ and $4F_4$ contained only two copies of the *LeNCED1* and lacked Tr-Ds element, confirmed by PCR. Plants $31F_4$ and $26F_4$ were homozygous for the *LeNCED1*. Error bars show the standard error of means of gene copy number in individual lines.

5.3.3 Genotype $59F_3$

5.3.3.1 A genotype with promising growth features with reduced g_s

This genotype originated from self-pollination of the parent plant $59F_2$. The F_2 plant had visually higher leaf angle from the stem and had the greyish leaf phenotype similar to the sp5 plants having low stomatal conductance (Table 5-6). The $59F_3$ seeds

germinated at the same frequency as the wild type without any chemical treatment. The early seedling establishment and growth of this plant was similar (data shown in Chapter-6) to wild type and no signs of interveinal flooding or chlorosis were observed.

Table 5-6: Leaf stomatal conductance (mmol m^{-2} s^{-1}) of $59F_2$ in comparison to the wild type and sp5 genotypes

GT	No.of plants	Mean g_s	*SEM	[1]p-value
****$59F_2$**	1	290.5	33.4	n/a
wild type	6	529.5	47.5	0.01
*****sp5**	6	205.9	31.7	

* represent standard error of means, calculated from multiple g_s values obtained from each plant.
**Genotype $59F_2$ germinated on water alone
***sp5 germinated on 3.28 μM norflurazon
[1]p-value indicates the data analysis through t-test assuming equal variance.

5.3.3.2 Genotypic analysis of $59F_3$

PCR results obtained by using the primers *Ds1For2* and *notRev5* (Appendix II) for detection of the *Ds* element in the $59F_3$ family (accession AT1679) showed that, out of 50 plants tested, 14 had lost the *Ds* element while 36 still retained an insertion. Chi-square test was in agreement that this genotype was heterozygous for a single *Ds* locus (Table 5-7).

Table 5-7: Calculation of segregation ratio of *Ds element* in genotype $59F_3$ by using Chi square test.

GT	Number of plants observed	Number of plants expected	x^2_{calc}	$x^2_{0.05,1}$	Ratio 3:1
$59F_3$-Ds^+	36	37.5			
$59F_3$-Ds^-	14	12.5			
Total	***50***	***50***	***0.24***	***3.841***	yes

Where x^2_{cal}.was the calculated Chi Square value
$x^2_{0.05,3}$ was the value from Chi Square table at p,0.05 and 3 degrees of freedom

5.3.3.3 Southern Hybridization Analysis

Genomic DNA from randomly selected plants containing the *Ds* element, as determined by PCR, was analysed by Southern blot. A probe was prepared using the complete ORF of *LeNCED1* gene. Data showed that there were two transposition events in the $59F_3$ family (Figure 5-11); these were named as *Tr-Ds*-6 and *Tr-Ds*-7. In total, 29 plants from genotype $59F_3$ preselected for the *Ds* element through PCR were analysed using Southern blotting. The results showed that all plants contained *Tr-Ds*-6, *Tr-Ds*-7 and the two T-DNA-Ds bands. This suggested that the F_2 parent was homozygous for all these bands, and so the family is uninformative with respect to genetic linkage.

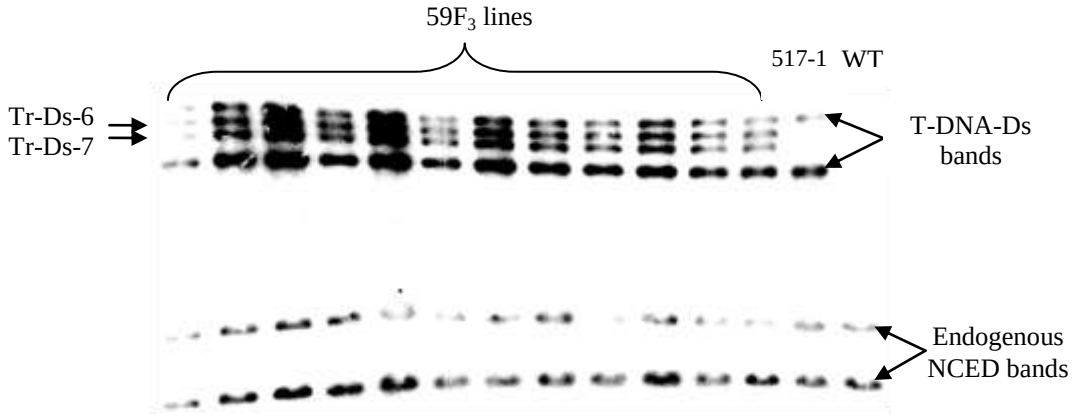

Figure 5.11. A Southern blot analysis of $59F_3$ plants preselected for the presence of *Ds* element. Blot hybridised with a probe containing complete ORF of *LeNCED1* gene. Where T-DNA-Ds represent bands inherited from 517-1 line, Tr-Ds shows the transposed bands.517-1 line and wild type were used as control.

5.3.3.4 Stomatal conductance measurements in genotype $59F_3$

To find out the effect of the *Tr-Ds* elements on the stomatal conductance, these plants were grouped in blocks each containing six plants with one wild type and one sp5 plant. There were six blocks in each screen. Data for the g_s was collected four times a day for two days. The g_s of $59F_3$ was significantly lower (40%) than the wild type plants but higher (20%) than sp5 plants (Figure 5-12). Also, the g_s of sp5 plants was significantly lower than the wild type plants under controlled environmental conditions, as expected ($P<0.001$).

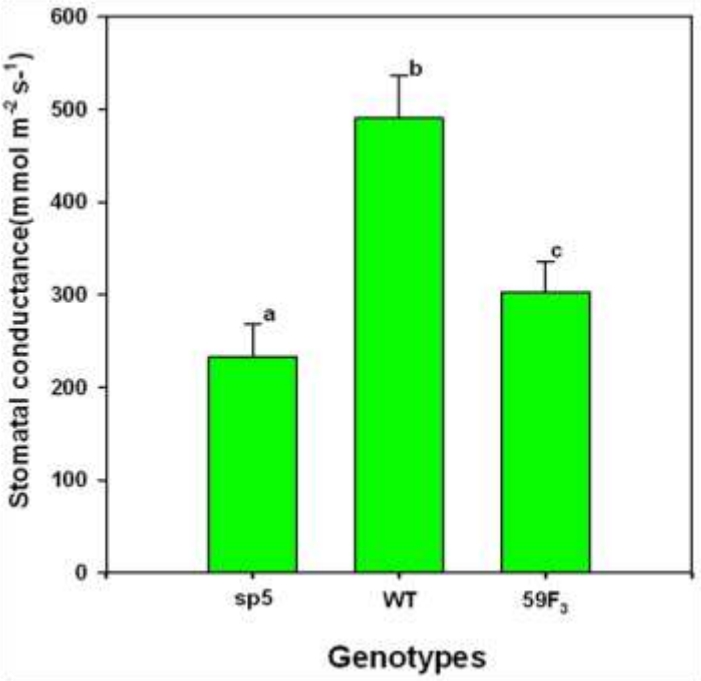

Figure 5.12. Means of stomatal conductance of genotype $59F_3$ under controlled environmental conditions analysed using ANOVA. Ailsa Craig $Tm2^a$(WT) and sp5 were used as control. Error bars represent the standard error of means. Different letters represent statistically significant differences at $P < 0.01$. n = 6

5.4 Discussion

Complex physiological parameters control the movement of stomata in response to environmental factors, and ultimately control conductance of the leaf and the rate of transpiration. These factors are wide ranging and include light (Yu et al., 2004, Kositsup et al., 2010), CO_2 (Robredo et al., 2007), VPD (Elsharkawy and Cock, 1984, Day, 2000, Pou et al., 2008), drought stress (Miyashita et al., 2005) and time of the day (Jarvis and McNaughton, 1986, Correia et al., 1997). Under glasshouse conditions, controlling most

of these parameters was not possible therefore all of the screening for the g_s was performed under controlled environmental conditions to reduce experimental variation.

The genotypes tested for g_s during the present study were randomly chosen with the main objective to select a line which had reduced g_s but no associated problems (e.g. poor seed germination and slow growth and establishment) (Thompson et al., 2000, Tung et al., 2008). Stomatal conductance can be a good indicator of plant water use efficiency (Earl, 2002, Thompson et al., 2007, Pou et al., 2008), hence, it was decided to select genotypes with reduced g_s to achieve higher WUE.

5.4.1 Genotype $116F_3$: Reduced g_s but issues with seed germination

Genotype $116F_3$ plants had a unique phenotype with visibly shorter internodes and reduced g_s which could be attributed to the transposition and reintegration of *Tr-Ds* elements in the genome (Hehl and Baker, 1989), but the plant growth remained apparently similar to the wild type except the seed germination. Southern blot analysis of plant population from $116F_3$ showed that the *Ds* element can also reintegrate at positions which are not linked (Vollbrecht et al., 2010), but most insertions are preferentially linked (Dooner and Belachew, 1989) 2-3 centimorgans from the original *Ds* element (Greenblatt, 1984). The results in this chapter revealed that the *Tr-Ds* elements (*Tr-Ds*-1, *Tr-Ds*-2 and *Tr-Ds*-3) were segregating through independent assortment (Table 5-2), and were not linked. Although on average the *Ds* positive plants had reduced g_s compared to wild type, the classification of the *Tr-Ds* elements showed no difference in the leaf g_s, (Figure 5-2) which could mean that the homozygous non-segregating *Tr-Ds*-1, or the original T-DNA-Ds insertions (discussed in section 5.3.1.3 of this chapter) or the accumulative effect of T-DNA-Ds and *Tr-Ds*-1 elements were

responsible for the reduced g_s. Unfortunaltly, it was not possible to determine the role of a particular locus controlling the reduction in g_s because the *Tr-Ds*-1 and the T-DNA-Ds were present in all lines of this family. This could be resolved by backcrossing. Further, poor seed germination in genotype $116F_3$, similar to the properties of already existing lines sp12 and sp5, led to the decision to do no further work was carried out on this line.

5.4.2 Genotype $102F_3$ exhibited a phenotype of putatively high ABA plant

The segregation ratio has demonstrated that all *Tr-Ds* elements in $102F_3$ genotype were closely linked as previously found in other examples (Burbidge et al., 1995, Semiarti et al., 2001, Tower et al., 1993) and segregated as a single complex locus (Table 5-3).

Several symptoms in common with previous reports of high ABA tomato lines, including interveinal flooding (Thompson et al., 2000, Tung et al., 2008), grayish leaf colour and a higher leaf angle from stem have been observed in this $102F_3$ genotype. It was also noticed that the plants of genotype $102F_3$ guttated at lower water vapour deficit (65% or above) (Figure 5-4). Exogenous ABA up to 10^{-4} M was used to collect plant sap from barley plants (Dieffenbach et al., 1980) , an early example where ABA was shown to result in leaf guttation from the hydathodes. Leaf guttation can be a result of high water potential and high root pressure. The leaf guttation by endogenous levels of ABA was previously observed in tomato (Thompson et al., 2007). The important new characteristic of genotype $102F_3$ was that seed germination was achieved on water

alone, whereas the seeds from lines over expressing *LeNCED1* using strong promoters (sp5 and sp12) showed delayed or norflurazon-dependent germination.

No signs of leaf interveinal flooding or chlorosis were observed at early establishment phase in $102F_3$ lines, but soon after transplantation into 10-cm pots (3 weeks old plants), it was observed that the seedlings started to exhibit leaf chlorosis and interveinal flooding (Figure 5-4). The reason for these symptoms appearing late in $102F_3$ might be a rapid root development after transplantation to bigger (10-cm) pots, increasing root: shoot ratio resulting in increased root pressure (Barrs, 1966, Palzkill and Tibbitts, 1977). The delay in root development appeared to be associated with an unexpected phenotype where the root meristem was arrested, and then allowed regrowth through production of laterals (Figure 5-3).

The upregulation of the *LeNCED1* gene in the *Tr-Ds* element during early development stage (3-week-old plants) causing reduced g_s and high hydraulic conductivity might be responsible for filling the intercellular spaces in the mesophyll tissues with the sap (Tung, et al., 2008; Thompson, et al., 2000). Plants with reduced g_s had more interveinal flooding and chlorosis on the lower older leaves only. This could be visible in sectors partitioned by leaf veins. Sectoring might be due to the heterobaric tomato leaf anatomy, as the substomatal air spaces are further divided by leaf veins or bundle sheaths which connect the lower epidermis to the palisade tissues. This structure stops the free flow of sap through the substomatal air spaces. It is hypothesized that the leaf chlorosis might be due to the damage caused to photosynthetic machinery including the plastids and thylakoid membranes as a result of interveinal flooding.

These results were highly consistent with the idea that the genotype $102F_3$ had constitutively high ABA contents (Thompson, et al. 2000) which might be due to the

transposition of the *Tr-Ds* element near an enhancer region resulting in increased expression of the transposed gene. Further analysis has been carried out to confirm these findings in Chapter-6.

5.4.3 Genotype $59F_3$ exhibited excellent growth features with a reduced g_s

By careful breeding programme, it is possible to achieve plants with high WUE_p under the influence of endogenous ABA. The results obtained from g_s data and visual observation of plant growth habit suggested that the genotype $59F_3$ presents excellent features both in terms of seed germination, seedling establishment and further plant growth and development together with reduced stomatal conductance. Southern blot data (Figure 5-11) revealed two unique transposition events in this genotype. Both loci were linked and were segregating in the population 3:1.This genotype therefore provides an excellent resource with potentially high WUE_p and high productivity (discussed in chapter -6).

5.4.4 Concluding remarks

The genotypes $102F_2$ and $59F_2$ showed promising indication of being high WUE_p exclusively determined by their reduced g_s compared to the wild type plants. Although genotype $116F_3$ had reduced g_s, but it had issues with seed germination which was against the objectives of achieving a high WUE_p without any problems associated with seed germination. Hence, it was decided not to include $116F_3$ for further experimentation. The genotypes $59F_2$ and $102F_2$ were thoroughly evaluated on the basis of their physiology, growth and development along with their gravimetric WUE_p. This has been described in the next chapter.

Chapter-6 Detailed phenotypic analysis of the selected genotypes with novel transposed *Ds* elements expressing *LeNCED1* gene

To evaluate the role of the *Ds* elements expressing *LeNCED1* gene in the modulation of ABA synthesis and its subsequent effect(s), more detailed experiments were designed which included genotypes $102F_2$ and $59F_2$. These genotypes were selected due to their interesting phenotype observed previously (details present in Chapters 4 &5). The key traits studied in this Chapter include the relative growth rate (RGR), net assimilation rate, total leaf area, bulk leaf ABA [ABA_{bl}], ABA concentration in the xylem sap [ABA_{xyl}], above ground plant biomass and plant water use efficiency (WUE_p). The correlation amongst these traits was also investigated.

6.1 Experimental Design

6.1.1 Plant material used in the experiments

Three F_3 lines, namely, $116F_3$, $102F_3$ and $59F_3$ were assessed for seed germination, plant establishment, further growth and leaf g_s in Chapter-5 sections 5.3.1, 5.3.2 and 5.3.3, respectively. Although genotype $116F_3$ had reduced g_s compared to the wild type plants, this genotype had an issue of slow seed germination (detailed in Chapter-5, section 5.3.1). The rate of seed germination could only be improved by using norflurazon (NZ). No further experimental work was carried out on genotype $116F_3$ because it did not comply with the objectives of this study 'to produce genotypes with good seed germination, establishment and growth rate with high WUE'.

Furthermore, the root growth was arrested soon after the seed germination in the F_4 progeny of genotype $102F_3$. This was caused by a recessive allele that segregated in

the progeny by the ratio of 3:1. A line that had lost this recessive gene was selected and the $102F_5$ generation (accession AT2524) was used during the experiments described in this chapter. For the plant $59F_2$, F_4 progeny, from two rounds of selfing, were used during the experiments detailed below.

As previously described in Chapter-1, the tomato lines transformed with *sAc* and T-DNA-Ds elements were in Money Maker and Ailsa Craig background, respectively. This was a potential source of genetic variation which could influence plant physiology (g_s, ABA contents and WUE_p). For this reason, populations from the $59F_4$ (accession AT2516) and $102F_5$ (accession AT2524) test genotypes were used which were segregating for the *Ds* element locus (3:1) so that plant with or without the *Ds* element, but with a randomized mixed Money Maker/Ailsa Craig background could be compared. The lines that have lost the *Ds* element by segregation are described as $59F_4$ (*Ds*-) and $102F_5$ (*Ds*-) and those that retain it are named $59F_4$ (*Ds*+) and $102F_5$ (*Ds*+). In addition to the *Ds*(-) and *Ds*(+) plants, genotype sp5 were included as a high ABA control.

Three separate experiments were conducted in glasshouse conditions under natural light. Experiment-1 and experiment-2 were conducted during March to May, 2010, while experiment-3 was conducted during June-August 2010. During all of these experiments, the genotypes were evaluated for growth starting from seed germination.

In experiment-1, the growth and WUE_p of genotype $102F_5$ (*Ds*+) was compared with the high ABA genotype sp5 and with $102F_5$ (*Ds*-) plants. These plants were grouped in six blocks and each block contained two plants from genotype $102F_5$ (*Ds*+), and one each from sp5 and $102F_5$(*Ds*-) genotypes. In experiment-2, genotype $59F_4$(*Ds*+) was compared with $59F_4$(*Ds*-) and sp5 plants for its growth, development and

gravimetric water use. Plants were randomly distributed in seven blocks, each block containing 2 plants from genotype $59F_4$(*Ds*+) and one plant each from sp5 and $59F_4$(*Ds*-) genotype.

6.1.2 Statistical design and Analysis

All experiments were designed according to randomized complete block design (RCBD). Mean values obtained from each plant in experiment-1 & 2 were analysed with Genstat V.12 (VSN International Ltd. Oxford, UK) by using Analysis of Variance (ANOVA). In experiment-3, which involved sequential harvests, the design was unbalanced and could not be analysed by comparing the means obtained from each plant. To overcome this complication, mean values from each genotype within a block were calculated followed by data analysis by using ANOVA.

6.2 Results

6.2.1 Experiment-1: Genotype $102F_5$

6.2.1.1. Growth analysis

Plants from genotype $102F_5$(*Ds*+), $102F_5$(*Ds*-) and sp5 were grown in the glasshouse. Gravimetric measurements of water use were started in 21 day old plants and continued for 27 days. Data from various plant traits were recorded at the end of the experiment. Leaf area (LA), number of fully expanded leaves, petiole length (cm), plant height (cm) and leaf angle of first fully expanded leaves from stem were recorded at the time of harvest (48 DAG). Statistical analysis of data revealed significant differences in some of the above-mentioned parameters (Table-6-1).

Leaf area (LA) in $102F_5$(*Ds*+) plants (2463 cm^2) was 33% less than the $102F_5$(*Ds*-) plants. Whereas, in sp5 plants was 13 % greater than $102F_5$(*Ds*-) and 46% more than $102F_5$(*Ds*+) plants (*P < 0.001*). (Table 6-1, *P<0.05*). There was no significant difference in number of fully expanded leaves (Table 6-1) in any of the genotypes used. Petioles in $102F_5$(*Ds*+) plants (36.4 cm) were significantly longer (*P < 0.04*) than for $102F_5$(*Ds*-) with petiole length of 33.2 cm. It was observed that sp5 plants were taller than $102F_5$(*Ds*+) plants (*P<0.001*) but there was no significant difference between sp5 plants and $102F_5$(*Ds*-) plants, with plant height 72.73 cm followed by 69.21 cm and 50.72 cm in sp5, $102F_5$(*Ds*-) and $102F_5$-*Ds*+ plants, respectively. The highest RGR (0.172 g g^{-1} day^{-1}) was recorded in sp5 plants higher than $102F_5$(*Ds*+) and $102F_5$(*Ds*-) plants with RGR 0.159 and 0.134 g g^{-1} day^{-1}, respectively (Table 6-1). The results obtained indicated statistically significant variation (*P<0.001*) in genotype's ability to gain dry weight over a period of 26 days (total duration of the gravimetric experiment). Maximum DW was gained by sp5 (36.63 g). This was 24% higher than the $102F_5$(*Ds*-) and 58% higher than $102F_5$(*Ds*+) plants.

For the leaf angle of the first fully expanded leaf from the stem (measuring *below* the leaf), sp5 plants had the higher leaf angle (117^o) statistically not different from $102F_5$(*Ds*+) plants (116^o). Lower leaf angle (86.5^o) was recorded in the $102F_5$(*Ds*-) plants. This was significantly lower than sp5 and $102F_5$(*Ds*+) genotypes at *P<0.05* (Table 6-1).

6.2.1.2 WUE_p

Genotype $102F_5$(*Ds*+) had minimum transpiration losses (1.92 kg H_2O), 65% less than $102F_5$(*Ds*-) and 70% less than sp5 plants under glasshouse conditions over the three weeks. Genotype $102F_5$(*Ds*+) had the greatest WUE_p, 61% higher (8.28g DW kg^{-1} H_2O), than the $102F_5$(*Ds*-) plants (*P<0.001*), while genotype sp5 had 12% higher WUE_p (5.81g DW kg^{-1} H_2O) compared to the $102F_5$(*Ds*-) plants (5.09g DW kg^{-1} H_2O).

Table 6-1: Effect of *LeNCED1* overexpression on plant growth and water use efficiency in genotype $102F_5$

		Genotypes				
Replications	**12**	**6**	**6**			
	$102F_5$(*Ds*+)	**$102F_5$(*Ds*-)**	**sp5**	***P-value***	***LSD* (min)**	**LSD (max-min)**
Leaf Area (cm^2)	2463.2^c	3980^b	4530.3^a	*<0.001*	441.28	382.16
No. of fully expanded leaves	10.25^a	11.33^a	10.83^a	*0.053*	1.019	0.882
Petiole length (cm)	36.42^a	33.17^b	38.67^a	*0.041*	3.124	2.706
Plant Height (cm)	50.72^b	69.21^a	72.73^a	*<0.001*	6.91	5.99
Leaf Angle (Degrees)	116^a	86.5^b	117^a	*<0.001*	3.154	2.731
Initial biomass (g dry wild type)	0.242^c	0.679^a	0.457^b	*<0.001*	0.209	0.1806
Biomass produced (g dry wild type)	15.59^c	28.19^b	36.63^a	*<0.001*	4.608	3.991
Relative growth rate (g g^{-1} day^{-1})	0.134^c	0.159^b	0.172^a	*<0.001*	0.003	0.002
Transpiration (Kg H_2O)	1.92^c	5.53^b	6.34^a	*0.001*	0.382	0.331
WUE_p (g dry wild type kg^{-1} H_2O)	8.28^a	5.09^c	5.81^b	*<0.001*	0.658	0.571

Note: Different letters in the same row indicate statistically significant differences in a variate *at LSD* 5% or less.

P values and LSD (least significant difference) values were obtained from ANOVA (5%). $102F_5$(*Ds*+), genotype containing the *Ds* element; $102F_5$(*Ds*-) genotype that lost the *Ds* element through segregation. Plant transpiration was measured by using gravimetric method over a period of 28 days. Only aboveground biomass was determined over the same period. Initial biomass was determined at the beginning of this experiment i.e. 21 days old plants.

6.2.2 Experiment-2: Comparison between various growth features in genotype $59F_4$(*Ds*+)

Plants from genotype $59F_4$(*Ds*+), $59F_4$(*Ds*-) and sp5 were grown in the glasshouse. Gravimetric measurements of water use were started in 16 day old plants and continued for 28 days. Data from various plant traits were recorded at the end of the experiment.

6.2.2.1 Growth analysis

Data obtained from experiment-2 (Table 6-2) revealed statistically significant differences in plant LA (*P<0.004*). There was no difference in the LA of $59F_4$(*Ds*+) and $59F_4$(*Ds*-) plants with LA of 4323cm^2 and 3883cm^2, but the LA of sp5 (2702 cm^2) was the lower (*P<0.05*). The total LA was 28% more in $59F_4$(*Ds*-) and 21% more in $59F_4$(*Ds*+) plants when compared to sp5 genotype. The number of fully expanded leaves was not quite statistically different at the 5% level (*P=0.052*) (Table-2). Similarly, there were no significant differences in the genotypes for petiole length, although sp5 had slightly longer petioles (Table 6-2).

There were significant differences in plant height amongst different genotypes (*P<0.002*). The $59F_4$(*Ds*-) plants had maximum plant height (66.1 cm) but statistically these were not different from $59F_4$(*Ds*+) plants (64.6 cm). The genotype with no *Ds element* i.e. $59F_4$(*Ds*-) gained maximum DW (31.15g) significantly higher than all other genotypes (*P < 0.05*; Table 6-2). It was followed by genotype $59F_4$(*Ds*+) (28.29g), whereas sp5 plants had minimum DW (18.77g). So there was 40% less DW in sp5 plants. When compared to $59F_4$(*Ds*-) plants, $59F_4$(*Ds*+) plants had 10% less above ground DW. Further, RGR data showed no differences between $59F_4$(*Ds*+) and

$59F_4$(*Ds*-) plants but both of these genotypes were, statistically higher than sp5 (0.165 g g^{-1} day^{-1}), as shown in Table 6-2.

Leaf angle showed significant differences between genotypes (Table 6-2). Maximum leaf angle (115.5^o) was recorded in sp5 plants, significantly higher than both $59F_4$(*Ds*+)and $59F_4$(*Ds*-) plants (*P<0.05*), whereas, minimum leaf angle (86.8^o) was recorded in the $59F_4$(*Ds*-) plants which was statistically similar to the plants containing the *Ds* element.

6.2.2.2 WUE_p

The lowest transpiration (2.83 Kg H_2O) was recorded in genotype sp5, significantly lower than all other genotypes (*P <0.001*), and $59F_4$(*Ds*-) plants had significantly higher rate of transpiration than $59F_4$(*Ds*+) at 5.17 and 4.51 Kg H_2O, respectively. Total transpiration in sp5 plants and $59F_4$(*Ds*+) was 46% and 13% less, respectively, in comparison to $59F_4$(*Ds*-) plants. Genotype sp5 was 10% more efficient in its water use than $59F_4$(*Ds*-); $59F_4$(*Ds*+) was only 4% better in terms of WUE_p when compared to the $59F_4$(*Ds*-) plants, but it was significantly different (*P<0.05*, Table 6-2). The highest WUE_p (6.63 g DW kg^{-1} H_2O) was calculated for sp5 plants; higher than for $59F_4$(*Ds*+) and $59F_4$(*Ds*-) plants which had WUE_p values of 6.27 and 6.02 g DW kg^{-1} H_2O, respectively.

6.2.3 Experiment-3: A comparative study of growth, development and WUE_p of $102F_5$ and $59F_4$

In this experiment genotypes $102F_5$(*Ds*+), $59F_4$(*Ds*+) and the respective control plants, $102F_5$(*Ds*-) , $59F_4$(*Ds*-), were grown simultaneously to confirm the results obtained during experiment-1 & 2. Plants of sp5 were also included in this experiment to confirm the results obtained during the previous studies and compare the WUE_p and other parameters with a standardised high WUE_p plant . It was predicted that the plants grown during June to August should have more available sunshine compared to the plants grown during March to May (Appendix-IV). This will have a direct influence on plant transpiration rates which are driven by solar heat gain (Ribeiro et al., 2006), further, sunlight stimulates stomatal opening to allow CO_2 fixation. Hence, there may be different sizes of genotype effect for growth and WUE_p at different times of the year.

This experimental set up consisted of randomized complete block design with three 24-module (P-24) trays constituting one block, with all five genotypes randomized in each block. During this experiment, more than 150 seeds from each genotype were germinated on moist (dH_2O only) filter papers in Petri dishes. The effect of germination was removed from establishment experiment by synchronization of germination. fOnly seed germinating on the same day were included in this experiment. The germinated seeds were transferred to Levington's F2s (peat based compost containing sand). Soon after the cotyledon emergence, the seedlings were photographed to calculate the rate of leaf area expansion on a daily basis (method described in chapter-2).

Table 6-2: Effect of *LeNCED1* overexpression on plant water use efficiency in genotype $59F_4$.

	Genotypes					
Replications	**14**	**7**	**7**		**ANOVA**	
	$59F_4$-*Ds*+	**$59F_4$-*Ds*- (**wild type**)**	**sp5**	***p value***	***LSD* (min rep)**	***LSD* (max-min rep)**
Leaf Area (cm^2)	3883^a	4323^a	2702^b	*0.004*	912.7 min rep	790.4 max-min
No. of fully expanded leaves	8.78^a	9.14^a	7.81^a	*0.052*	1.312 min rep	1.137 max-min
Petiole length (cm)	40.68^a	40^a	41.36^a	*0.332*	1.848 min rep	1.601 max-min
Plant Height (cm)	64.6^a	66.1^a	53.4^b	*0.002*	6.85 min rep	6.45 max-min
Leaf Angle (Degrees)	91.5^b	86.8^b	115.5^a	*<0.001*	4.734 min rep	4.099 max-min
Initial biomass (g dry wild type)	0.31^a	0.36^a	0.18^b	*0.003*	0.087 min	0.061 max-min
Biomass produced(g dry wild type)	28.29^b	31.15^a	25.39^c	*<0.001*	3.294 min	2.852 max-min
Relative growth rate ($g\ g^{-1}\ day^{-1}$)	0.188^a	0.192^a	0.165^b	*<0.001*	0.002 min rep	0.0015 max-min
Transpiration (kg H_2O)	4.51^b	5.17^a	3.83^c	*<0.001*	0.382 min	0.331 max-min
WUE_p (g dry wild type kg^{-1} H_2O)	6.27^b	6.02^c	6.63^a	*0.01*	0.274 min rep	0.237 max-min

Note: Different letters in the same row indicate statistically significant differences in a variate *at LSD* 5% or less. P values and LSD (least significant difference) were obtained from ANOVA (5%). $59F_4$-*Ds*+; genotyping containing the *Ds* element; $59F_4$-*Ds*-; lost the *Ds* element through segregation. Plant transpiration was measured by using gravimetric method over a period of 28 days. Only aboveground biomass was determined over the time period. Initial biomass was determined at the beginning of this experiment i.e. 16 DAG.

6.2.3.1 Sequential Harvest: detailed

Plants were sequentially harvested every 14 days up to 56 days in order to assess the consistency of the phenotypic differences measured previously (in experiment 1&2), and to obtain higher resolution in time. The hypothesis that the high ABA genotypes have slow initial growth due to higher sensitivity towards this phytohormone followed by a rapid boost in growth was tested in the subsequent sections described below.

In order to investigate this hypothesis, plant growth data was collected and ABA contents were measured at various harvest intervals from both leaves and xylem sap. During the first harvest, the plants from the segregating $102F_5$ and $59F_4$ families were not yet genotyped; hence 40 plants were sampled from each genotype to allow for a sufficient number of plants of each category. In total, 20 sp5 plants were harvested across four blocks. Five plants were harvested from each genotype across each block during the subsequent harvests (i.e. 28 DAG, 42 DAG and 56 DAG).

6.2.3.2 Genotype analysis

Genotyping of both selected lines was carried out through PCR. Primers used for this purpose were *Ds*1For2 and *notRev5* (Appendix I). Out of 79 plants tested in genotype $102F_5$, 56 plants contained the *Ds* element and 23 had lost the *Ds* element through segregation. A Chi-square test ($\chi^2 = 0.60$ at $P < 0.05$) showed no significant deviation from segregation at 3:1. The segregation ratio in $59F_4$ plants was also found to be 3:1. This was confirmed by using the Chi-square test ($\chi^2 = 0.24$ at $P < 0.05$). Out of 61 plants tested, 44 plants contained the *Ds* element while 17 had lost it through segregation.

6.2.3.3 Phenotypic analysis at various harvest intervals

No difference between the genotype $59F_4$(*Ds*+) and $59F_4$(*Ds*-) could be recorded in any of the growth parameters studied (*P<0.05*). However, significant differences were recorded in $102F_5$(*Ds*+) and $102F_5$(*Ds*-) and sp5 have been described below.

6.2.3.3.1 Total leaf area (cm^2)

At 7 DAG, LA in genotype $102F_5$(*Ds*+) was 16% lower than $102F_5$(*Ds*-) (Figure 6-1.A), 11 and 13-cm^2 respectively. This difference was non-significant(*P* <0.05), (Figure 6-1.B). Genotype sp5 had the lowest LA (6-cm^2) (Figure 6-1 A & B), significantly smaller than $102F_5$(*Ds*+) and $59F_4$(*Ds*+). LA in sp5 was 45% and 65% lower than $102F_5$(*Ds*+) and $59F_4$(*Ds*+) plants, respectively (*P<0.001*).

At 14 DAG, genotype $102F_5$(*Ds*+) had 56% lower LA (23-cm^2) than $102F_5$(*Ds*-) plants with LA of 52cm^2. This was significantly different at *P<0.001* (Figure 6-1.A), Similarly, 28 DAG the LA of genotype sp5 had caught up with $102F_5$(*Ds*+) at this stage with LA of 161 and 149-cm^2, respectively (Figure 6-1.A). The genotype $102F_5$(*Ds*+) had nearly 53% less LA compared to $102F_5$(*Ds*-) (316-cm^2) plants, significantly lower (*P<0.001*) at this stage as well.

At 42 DAG, the LA in genotype $102F_5$(*Ds*+) was 1328-cm^2, but the LA of $102F_5$(*Ds*-) was 49% higher (*P < 0.001*) (Figure 6-1B). On the final harvest, 56 DAG, there was a significant increase in the LA of genotype $102F_5$(*Ds*+) which then had only an 11% less LA than $102F_5$(*Ds*-), with 5247 and 5888-cm^2 LA, respectively. However, it was still significantly lower than $102F_5$(*Ds*-) (*P<0.01*). At this final harvest the sp5 plants had the highest LA (7111-cm^2), 27% and 15% more than $102F_5$(*Ds*+) and $59F_4$(*Ds*+), respectively (*P<0.05*) (Figure 6-1. A & B).

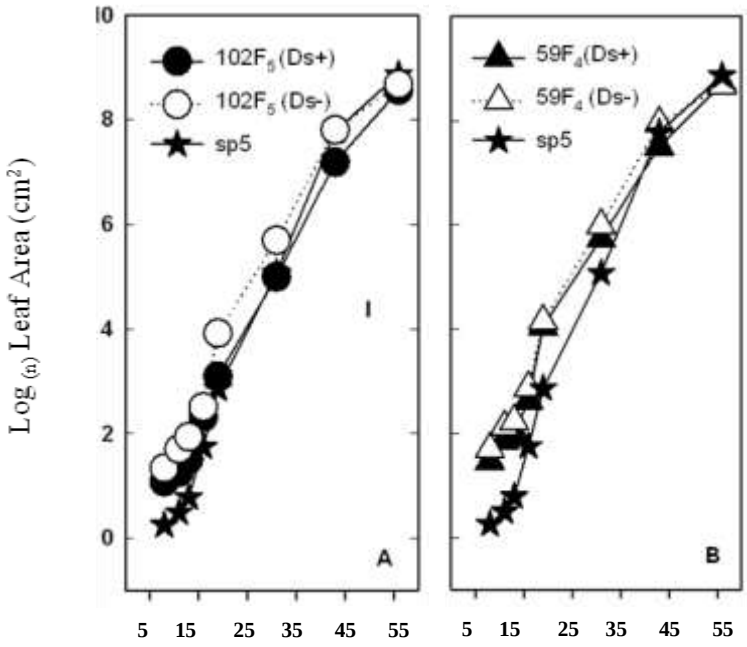

Figure 6.1. Changes in total leaf area of various genotypes measured at different growth stages. Where $Log_{(n)}$ shows the natural log of the total leaf area measured in cm^2. Panel A shows the log transformed data from genotype $102F_5$(*Ds*+/-) with sp5. Panel B shows the log transformed data from genotype $59F_4$(*Ds*+/-) with the same sp5 as shown in panel A. The genotypes $102F_5$(*Ds*+) and $59F_4$(*Ds*+) contained the Tr-Ds and T-DNA-*Ds* elements. Genotypes $102F_5$(*Ds*-) and $59F_4$(*Ds*-) had lost the T-DNA-*Ds* and Tr-Ds elements through segregation. Genotype sp5 was used as an additional control overexpressing *LeNCED1* transgene using Gelvin super promoter. The vertical floating bar indicates the *LSD* values at $P < 0.05$.

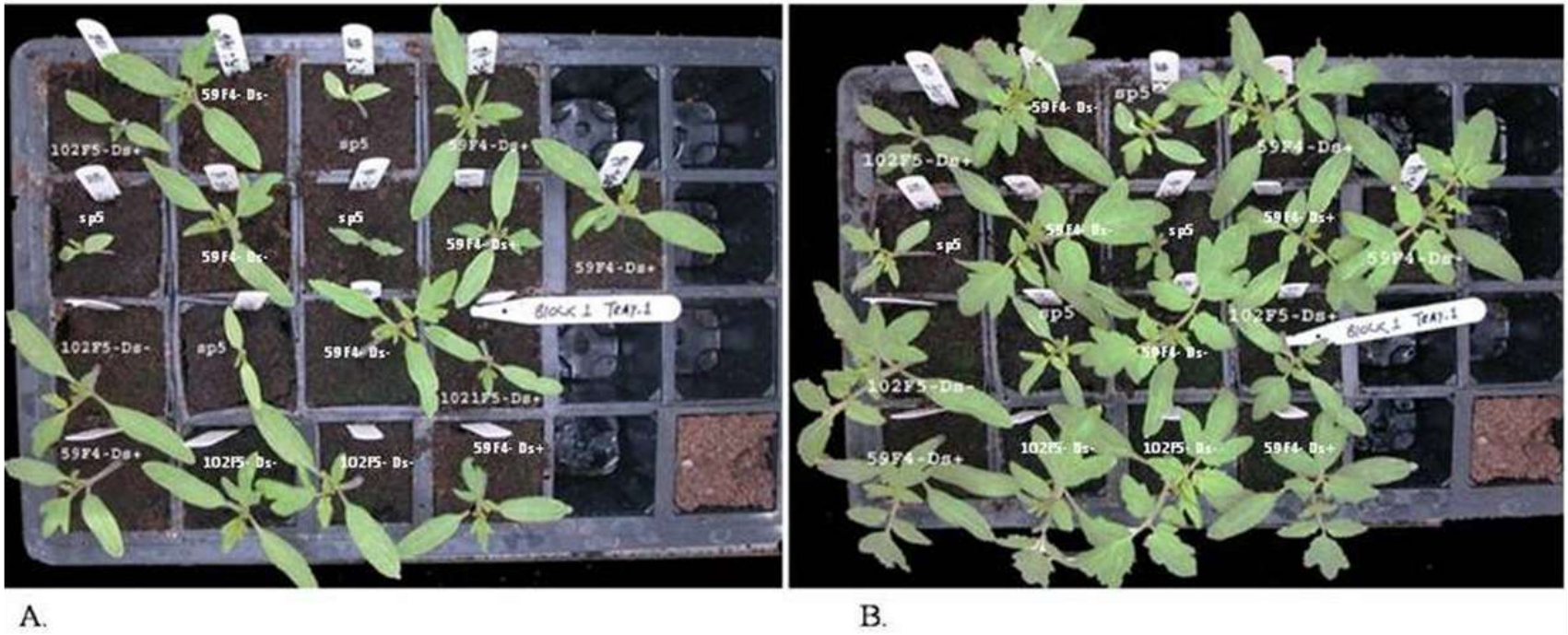

Figure 6.2. Effect of overexpression of *LeNCED1* gene on plant morphological traits. **A**. P-24 tray containing 7- days old seedlings from different genotypes. **B**. A P-24 tray containing 11-days old seedlings of different genotypes. Images were taken from above with a still camera from a fixed height. Genotype $102F_5$(*Ds*+) and $59F_4$(*Ds*+) contained the Tr-Ds and T-DNA-*Ds* elements. Genotypes $102F_5$(*Ds*-) and $59F_4$(*Ds*-) lost the T-DNA-*Ds* and Tr-Ds elements through segregation. Genotype sp5 was used as control as this genotype had known reduced g_s and high ABA.

6.2.3.3.2 Leaf area expansion rate (cm^2 day^{-1}):

Leaf area expansion rate (LER) was calculated from LA at each time point (i.e. the rate of increase in LA from one harvest point to another harvest). This showed statistically significant differences (*P<0.05*) between genotypes at various harvest intervals. However, no differernce was observed between $59F_4$(*Ds*+) and $59F_4$(*Ds*-) at *P<0.05*.

The LER in genotype $102F_5$ (*Ds*+) was significantly lower (1.59 cm^2 day^{-1}) than $102F_5$ (*Ds*-) plants (1.97 cm^2 day^{-1}) between 14-28 DAG (Figure 6-3.a), but became similar to $102F_5$ (*Ds*+) between 28 and 42 DAG, and then became significantly higher at the final harvest i.e. 42-56 DAG, ($102F_5$(*Ds*+), 1.37 cm^2 day^{-1}; $102F_5$ (*Ds*-), (0.87 cm^2 day^{-1}).

A comparison between genotype $102F_5$(*Ds*+) and $59F_4$(*Ds*+) showed that genotype $59F_4$(*Ds*+) had significantly higher LER (2.41 cm^2 day^{-1}) than $102F_5$(*Ds*+) (1.89 cm^2day^{-1}) between 5-14 DAG (Figure 6-3.b, *P < 0.01*), but at subsequent time points $102F_5$(*Ds*+) plants had higher LER. This was significant between 42-56 DAG.

The LER of sp5 at 14 DAG was lower than both wild type genotypes i.e. $59F_4$(*Ds*-) and $102F_5$(*Ds*-) although it was statistically not different (Figure 6-3.c). At the three later time points sp5 had a higher LER than $59F_4$(*Ds*-), and this was significant between 42-56 DAG (*P < 0.05*). Genotype $59F_4$(*Ds*+) had similar LER to $59F_4$(*Ds*-), except at the time of final harvest (Figure 6-3.d), when this genotype had significantly higher LER (1.26 cm^2 day^{-1}) than the $59F_4$(*Ds*-) plants (0.73 cm^2 day^{-1}).

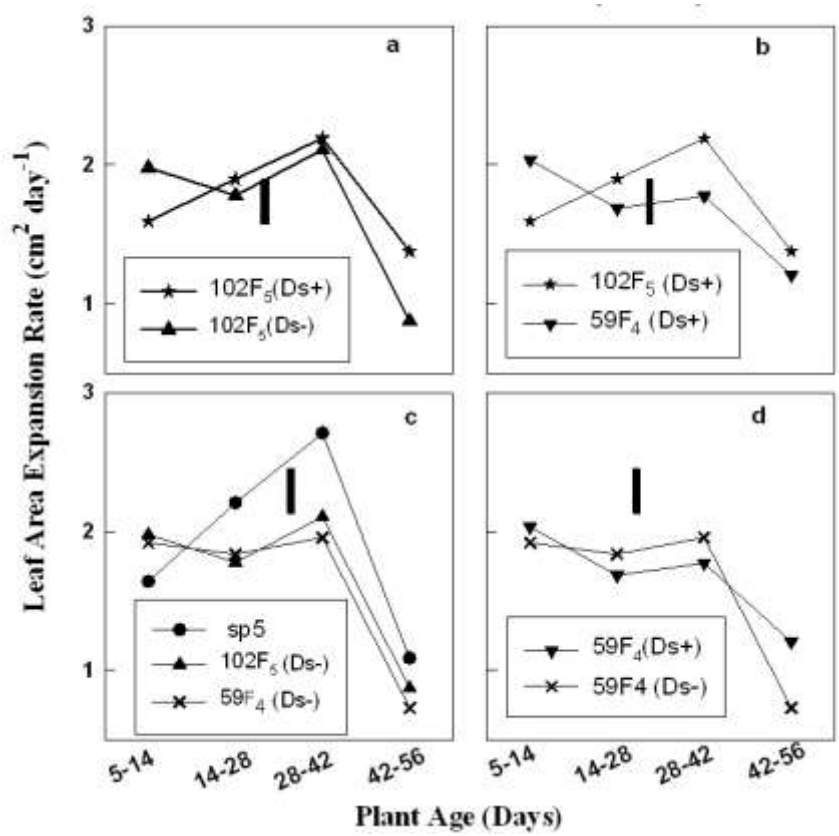

Figure 6.3. Leaf Area expansion rate (cm^2 day^{-1}) various genotypes at different harvest intervals. Genotype 102F$_5$(*Ds*+) and 59F$_4$(*Ds*+) contained the Tr-Ds and T-DNA-*Ds* elements. Genotypes 102F$_5$(*Ds*-) and 59F$_4$(*Ds*-) lost the T-DNA-*Ds* and Tr-Ds elements through segregation. Genotype sp5 was used as control over expressing *LeNCED1* transgene using Galvin Super promoter and was putatively high ABA line. Vertical floating bars indicate the LSD value at $p < 0.05$, where n=5.

6.2.3.3.3 Shoot dry weight (mg)

Above ground plant dry weight of the selected genotypes was log transformed (natural log) to normalize the data set for statistical analysis. Genotypes accumulated different above ground DW except 59F4(*Ds*+) and 59F$_4$(*Ds*-) at all harvest intervals. At 14 DAG, the genotype 102F$_5$(*Ds*+) had statistically lower DW (0.130 g) compared to the its wild type (0.323g) ($P < 0.01$) and sp5 plants had the smallest DW (0.092 g),

nearly 30% and 70% less than $102F_5$(*Ds*+) and $59F_4$(*Ds*+) plants, respectively (Figure 6-4.A & B).

The difference between genotype $102F_5$(*Ds*+) and its wild type further increased at 28 DAG, at this point genotypes $102F_5$(*Ds*+) had DW 0.713g significantly lower than $102F_5$(*Ds*-) plant with DW of 1.495g (*P<0.001*). The sp5 plants had the lowest DW at this harvest stage (0.620 g) shown in Figure-6-4.

At 42 DAG genotype $102F_5$(*Ds*+) had significantly lower DW (6.06 g) compared to $102F_5$(*Ds*-) (12.45 g) at ($P < 0.001$). At this stage the genotype sp5 accumulated significantly more above-ground DW compared to $102F_5$(*Ds*+), however, its DW was lower than $59F_4$(*Ds*+) and the two Ds- lines.

Similarly, 56 DAG, the minimum DW was recorded in genotype $102F_5$(*Ds*+) (32.39 g), 32% and 27% lower than $102F_5$(*Ds*-) and $59F_4$(*Ds*+) plants, respectively (Figure 6-4 A & B) (*P < 0.001*). There was a significant relative increase in the DW of sp5 during 14 days harvest interval (42-56 DAG). The DW of sp5 (54.53 g) was significantly higher than all other genotypes 56 DAG ($P < 0.001$) as shown in Figure-6-4 A & B).

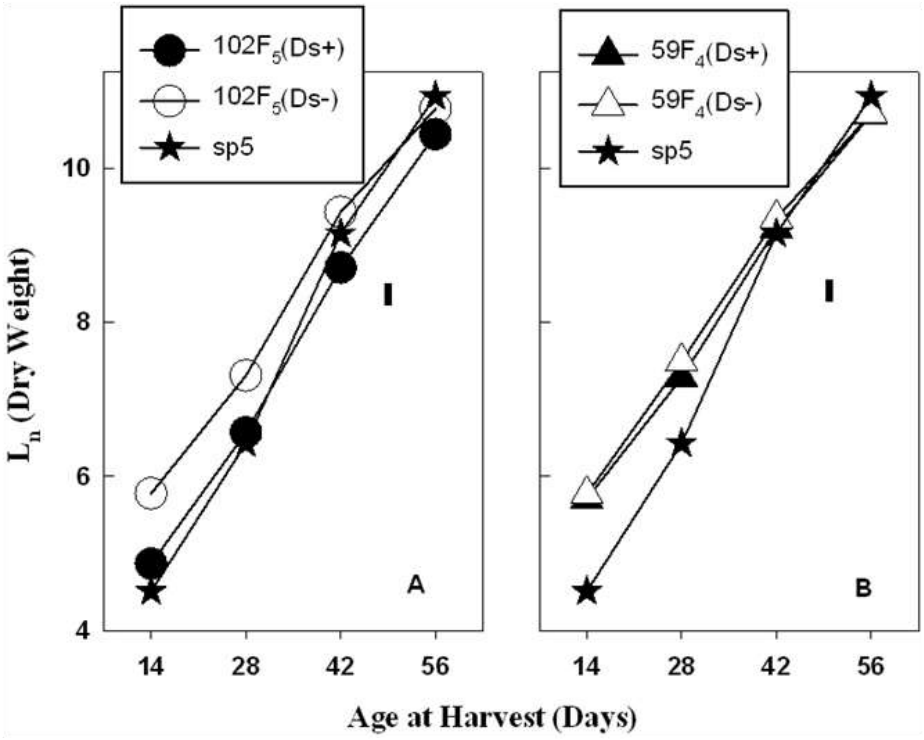

Figure 6.4. Plant dry weight (log transformed) of different genotypes at various harvest stages. Panel 'A' indicates log transformed dry weight of genotype $102F_5$(*Ds*+), $102F_5$(*Ds*-) and sp5; B, shows the log transformed data of genotype $59F_4$(*Ds*+), $59F_4$(*Ds*-) and sp5 at various harvest intervals. Genotype $102F_5$(*Ds*+) and $59F_4$(*Ds*+) contained the Tr-Ds and T-DNA-*Ds* elements. Genotypes $102F_5$(*Ds*-) and $59F_4$(*Ds*-) had lost the T-DNA-*Ds* and Tr-Ds elements through segregation. Genotype sp5 was used as control over expressing *LeNCED1* transgene using Galvin Super promoter and was putatively high ABA line. Vertical floating bars indicate the *LSD* value at p <0.05, n=5.

6.2.3.3.4 Net assimilation rate (g cm^{-2} day^{-1})

Net assimilation rate is the rate of dry matter production per unit of leaf area. Crops with higher NAR have higher photosynthetic efficiency, and hence it is an important plant trait from an agronomic point of view.

Results showed significant differences (P < 0.05) in genotypes for net assimilation rate at the later harvest intervals (Figure 6.5). However, $59F_4$(*Ds*+) and

$59F_4$(*Ds*-) were statistically similar. Maximum NAR (0.559 mg cm^{-2} day^{-1}) at 14-28 days interval was calculated in $102F_5$(*Ds*-) genotype and it was statistically similar to $59F_4$(*Ds*-) and $59F_4$(*Ds*+) plants with NAR values 0.537 and 0.503 mg cm^{-2} day^{-1}, respectively. Genotype sp5 had the lowest NAR (0.460 g cm^{-2} day^{-1}) followed by $102F_5$(*Ds*+) plants (0.471 mg cm^{-2} day^{-1}) and both genotypes were statistically similar 14-28 days interval.

There was a significant increase in NAR in genotypes $102F_5$(*Ds*+) and sp5 between 42-56 days. The highest NAR (0.777 g cm^{-2} day^{-1}) was calculated in sp5 plants, it was higher than all genotypes (*P<0.01*) except $102F_5$(*Ds*+) with NAR of 0.752 mg $cm^{-2}day^{-1}$. There was no difference in genotypes that lost the *Ds* element through segregation at 42-56 interval (Figure 6.5).

6.2.3.3.5 Plant relative growth rate (g g^{-1} day^{-1})

Relative growth rate (RGR) was statistically similar in both $59F_4$(*Ds*+) and $59F_4$(*Ds*-) at all growth intervals (Figure 6-6). At 0-14 days interval genotype $59F_4$(*Ds*-) had maximum RGR (0.141 g g^{-1} day^{-1}) which was statistically similar to $59F_4$(*Ds*+) and $102F_5$(*Ds*-). All the aforementioned genotypes had higher RGR compared to $102F_5$(*Ds*+) (0.113 g g^{-1} day^{-1}) and sp5 (0.107 g g^{-1} day^{-1}) at *P<0.01*. It was noticed that that the RGR was approximately two folds higher during 0-14 DAG compared to 28, 42 and 56 DAG.

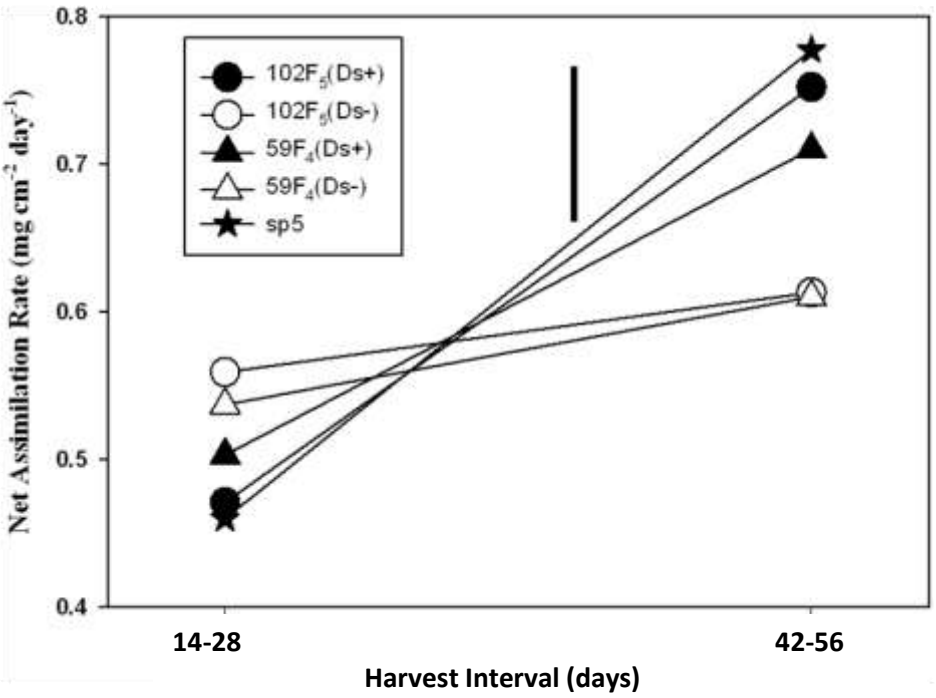

Figure 6.5. Net assimilation rate (g m^{-2} day^{-1}) calculated at two different stages in tomato plants grown under the glasshouse. Genotype $102F_5$(*Ds*+) and $59F_4$(*Ds*+) contained the Tr-Ds and T-DNA-*Ds* elements. Genotypes $102F_5$(*Ds*-) and $59F_4$(*Ds*-) lost the T-DNA-*Ds* and Tr-Ds elements through segregation. Genotype sp5 was used as control over expressing *LeNCED1* transgene using Galvin Super promoter and was putatively high ABA line. Vertical floating bars indicate the LSD value at p <0.05, where n=5.

For the 14-28 days interval the maximum RGR (0. 0.061 g g^{-1} day^{-1}) was recorded in sp5 plants. This was statistically higher than all other genotypes used in the experiment (P < 0.001). Minimum RGR (0.048 g g^{-1} day^{-1}) was recorded in genotype $102F_5$(*Ds*-) at 14-28 interval. The RGR of $102F_5$(*Ds*+) at this stage was statistically similar $102F_5$(*Ds*-).

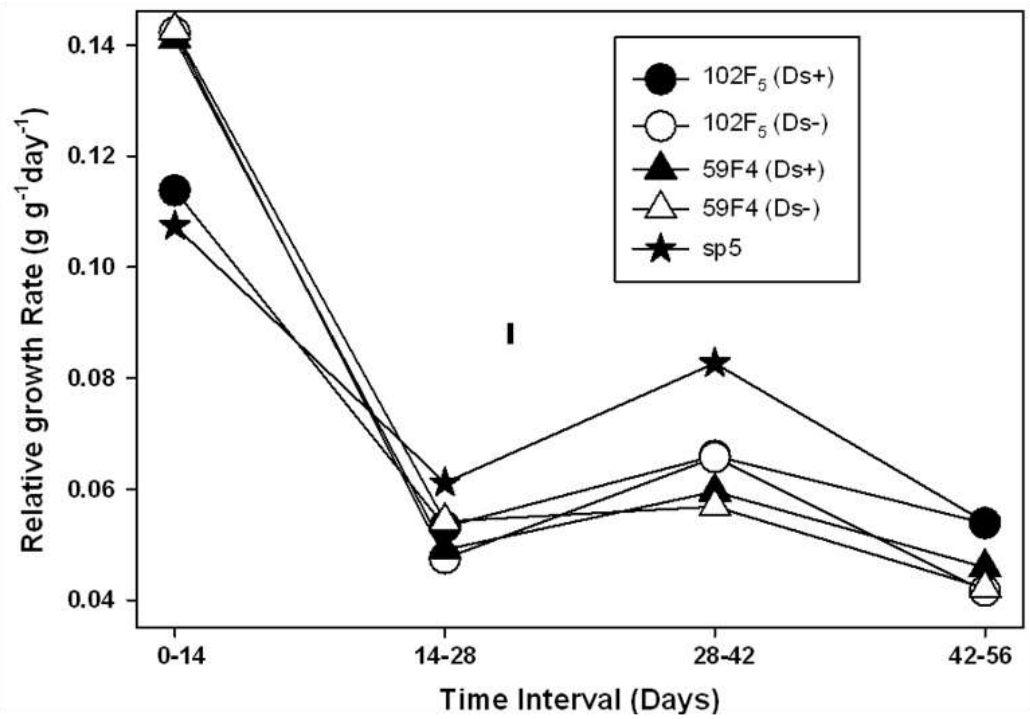

Figure 6.6. Relative growth rate (g g^{-1} day^{-1}) of different genotypes determined at various harvest stages. Genotypes $102F_5$(*Ds*-) and $59F_4$(*Ds*-) lost the T-DNA-*Ds* and Tr-Ds elements through segregation. Genotype sp5 was used as control over expressing *LeNCED1* transgene using Galvin Super promoter and was putatively high ABA line. Floating bar shows the LSD value at $p<0.05$, n=5.

At 28-42 days interval, genotype sp5 had the highest RGR (0.083 g g^{-1} day^{-1}) significantly higher ($P < 0.001$) than all other genotypes. There was no difference in genotype $102F_5$(*Ds*+) and $102F_5$(*Ds*-) during 28-42 days interval.

The RGR was generally at its lowest level during the 42-56 days interval, and in this case maximum RGR (0.054 g g^{-1} day^{-1}) was recorded in $102F_5$(*Ds*+) plants. This was significantly higher than $102F_5$(*Ds*-) plants at 42-56 days interval (Figure 6-6).

6.2.3.4 Growth traits at the time of final harvest (56 DAG)

6.2.3.4.1 Fractional biomass distribution

Genotype $102F_5$(*Ds*+) had higher biomass allocation to lamina (43%) compared to 40 % in $102F_5$(*Ds*-), but the difference was non-significant ($P < 0.05$). Also, no difference was found between genotype $59F_4$(*Ds*+) and $59F_4$(*Ds*-). The highest allocation of resources towards lamina (45%) was recorded in sp5 plants and this was statistically higher than all other genotypes apart from $102F_5$(Ds+) ($P < 0.01$).

The highest resource allocation to stem (31%) was $102F_5$(*Ds*-) plants (Table 6-3) and it was statistically similar to genotype $59F_4$(*Ds*+) and $59F_4$(*Ds*-). The lowest (26%) resources allocation to stem was recorded in genotype sp5 plants significantly lower (*P<0.001*) than all other genotypes except $102F_5$(*Ds*+).

The highest fraction of biomass towards petiole (27%) was allocated in genotype $102F_5$(*Ds*+) significantly higher from $102F_5$(*Ds*-) plants (Table 6-3). Maximum allocation to inflorescence (8%) was in $59F_4$(*Ds*-), statistically it was similar to genotype $59F_4$(*Ds*-) plants. However, $102F_5$(*Ds*+) plants had significantly lower resource allocation to the inflorescence (3%) compared to $102F_5$(*Ds*-) plants with 7% biomass allocated to the inflorescence (Table 6-3).

6.2.3.4.2 Petiole Length (cm)

Petiole length (cm) was significantly different ($P < 0.05$) between genotypes (Figure 6-7.A), however, $59F_4$(*Ds*+) and $59F_4$(*Ds*-) were statistically not different. The genotype $102F_5$(*Ds*+) plants had longer petioles (42.90 cm) than $102F_5$(*Ds*-) plants. However, the longest petioles were recorded in sp5 (46.30 cm) and this was significantly higher than all other genotypes (*P<0.05*).

6.2.3.4.3 Extent of leaf Epinasty

During the experiment, it was observed that plants from genotype $59F_4$(*Ds*+) and $59F_4$(*Ds*-) showed severe epinasty during noon time (Figure 6-8), when the rate of transpiration was maximum. The leaf epinasty was visually scored on each plant throughout plant growth and at various times of the day. Maximum score was recorded in $59F_4$(*Ds*+) plants (6.0) this was statistically similar to $59F_4$(*Ds*-) plants. Whereas, minimum leaf epinasty score was recorded in the genotype $102F_5$(*Ds*+) plants (1.5), which was significantly lower ($P < 0.001$) than plants from genotype $102F_5$(*Ds*-) shown in Figure 6-7.B.

Table 6-3: Fractional biomass distribution of various above-ground plant tissues in different genotypes

Replications	**12**	**4**	**12**	**4**	**8**	**LSD values**			**p-value**
Tissue/GT	**102F$_5$ *Ds*+**	**102 F$_5$ *Ds*-**	**59 F$_4$ *Ds*+**	**59 F$_4$ *Ds*-**	**sp5**	**min.rep**	**max-min**	**max.rep**	
Stem	0.27^{bc}	0.31^{a}	0.28^{b}	0.30^{ab}	0.26^{c}	0.026	0.021	0.015	*<0.001*
Lamina	0.43^{ab}	0.40^{b}	0.42^{b}	0.41^{b}	0.45^{a}	0.041	0.033	0.024	*0.002*
Petiole	0.27^{a}	0.22^{b}	0.23^{b}	0.21^{bc}	0.24^{b}	0.03	0.024	0.017	*<0.001*
Inflorescence	0.03^{b}	0.07^{a}	0.07^{a}	0.08^{a}	0.05^{ab}	0.036	0.029	0.021	*0.004*

Note: Different letters in the same row indicate statistically significant differences in a variate *at LSD* 5% or less.

P values and LSD (least significant difference) values were obtained from ANOVA. The plants were harvested 56 days after germination. Genotypes 102F$_5$(*Ds*-) and 59F$_4$(*Ds*-) lost the T-DNA-*Ds* and Tr-Ds elements through segregation. Genotype sp5 was used as control over expressing *LeNCED1* transgene using Galvin Super promoter and was putatively high ABA line, n=5.

6.2.3.4.4 Time required to Flowering

The $59F_4$(*Ds*-) plants started flowering the earliest (48 DAG), statistically similar to $59F_4$(*Ds*+), $102F_5$(*Ds*-) and sp5 plants which took 49, 49 and 50 DAG, respectively (Figure 6-7.C). The $102F_5$(*Ds*+) plants took significantly more time (54 DAG) to reach flowering stage.

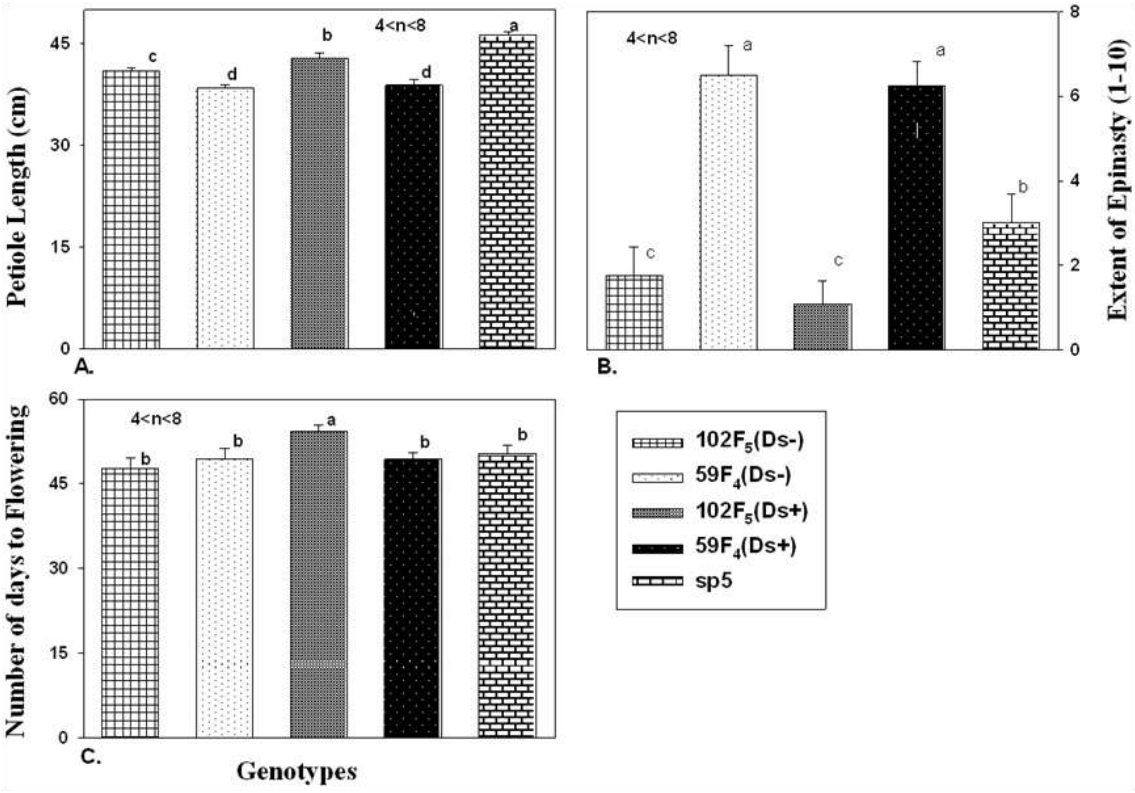

Figure 6.7. Plant growth and developmental traits in genotypes grown during the experiment. **A**. Average petiole length (cm) of fully expanded leaves in different genotypes **B**. Extent of epinasty in 56 days old plants grown under well watered conditions. **C.** Number of days required for plants to reach the reproductive phase in different genotypes. Different letters indicate statistically significant differences at $P<0.05$. Error bars show the standard error of means.

6.2.3.5 Water Relations of Different Genotypes

Leaf water potential (Ψ_l), leaf osmotic potential (Ψ_s) and leaf turgor pressure (Ψ_p) were determined at the time of final harvest (i.e. 56 DAG). As there was no difference shown by $59F_4$(*Ds*-) and $102F_5$(*Ds*-) genotypes, data obtained from these genotypes was pooled, hence, these genotypes have been referred as wild type. Further, genotype $59F_4$(*Ds*+) and $59F_4$(*Ds*-) were statistically similar for these parameters.

6.2.3.5.1 Leaf Water Potential (MPa)

Statistical analysis of leaf water potential (Ψ_l) revealed significant differences among the genotypes (Figure 6-8.A) at $P<0.001$. Maximum Ψ_l (-0.72 MPa) was in $102F_5$(*Ds*+) plants, and this was statistically similar to sp5 plants (-0.78 MPa) but significantly higher than $59F_4$(*Ds*+) and wild type plants. The lowest Ψ_l was in wild type plants (-1.07 MPa) shown in Figure 6-9.A.

Figure 6.8. The extent of leaf epinasty in different genotypes. **A.** Shows an epinastic leaf in genotype $59F_4$(*Ds*+) plant. **B.** Shows a similar age leaf from genotype $102F_5$(*Ds*+) plant, with no sign of epinasty.

6.2.3.5.2 Osmotic Potential (MPa) and Turgor Pressure (MPa)

Leaf osmotic potential (Ψ_s) was measured with an osmometer (detail in chapter-2), but no statistical differences between the genotypes were found (Figure 6-9-B). The results from leaf turgor pressure (Ψ_p) revealed that the genotypes sp5 and $102F_5$(*Ds*+) had the highest Ψ_p values (0.52 MPa), and these were statistically different ($P < 0.001$) from wild type and $59F_4$(*Ds*+) plants. Further, genotype $59F_4$(*Ds*+) also had higher Ψ_p compared to the wild type plants (Figure 6-9.C) at $P < 0.001$.

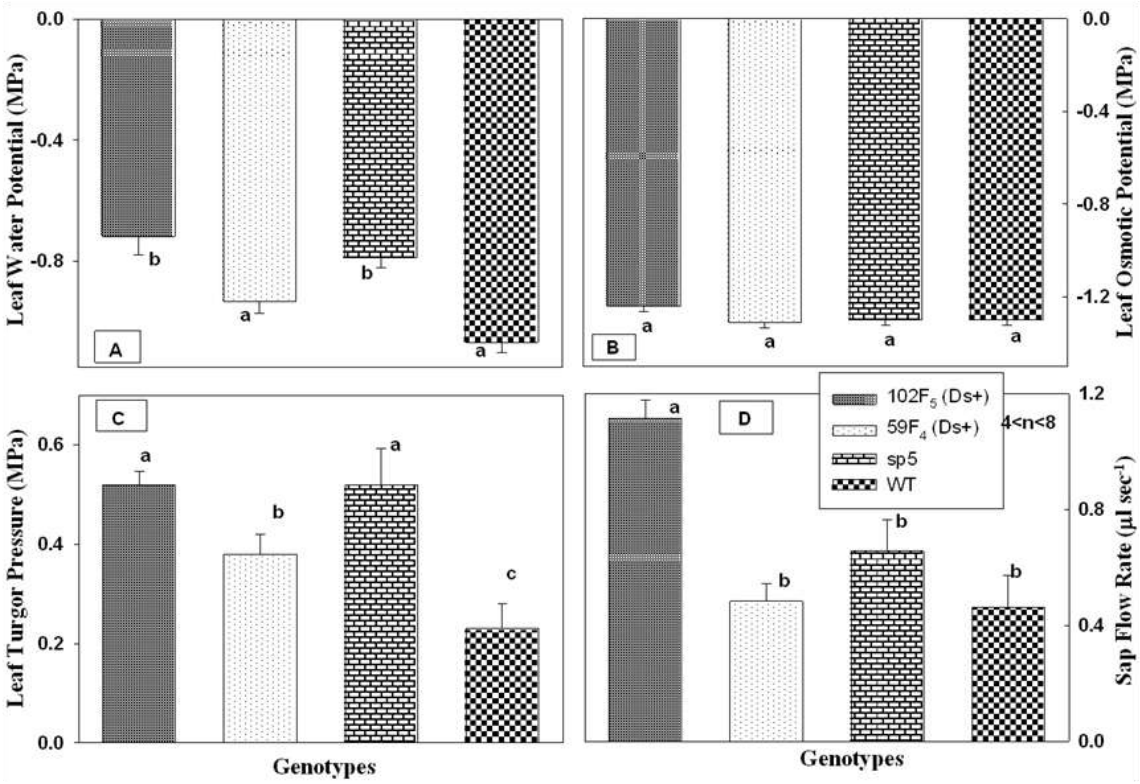

Figure 6.9. Leaf Water potential (MPa), Osmotic potential (MPa),Turgor Pressure (MPa) and sap flow rate (µl s^{-1}) of 56 days old plants from various genotypes. Genotype $102F_5$(*Ds*+) and $59F_4$(*Ds*+) contained the Tr-Ds and T-DNA-*Ds* elements. Genotypes $102F_5$(*Ds*-) and $59F_4$(*Ds*-) lost the T-DNA-*Ds* and Tr-Ds elements through segregation and data from $102F_5$(*Ds*-) and 59F4(*Ds*-) was pooled as it was statistically not different. Different letters on the same graph indicate statistically significant differences between the genotypes at $p < 0.05$. Error bars show the standard error of means, where $4 \leq n \leq 8$.

6.2.3.5.3 Sap flow rate (µl s^{-1})

Sap flow rate was higher in $102F_5$(*Ds*+) detopped plants (1.12 µl s^{-1}) in comparison to the other genotypes (Figure 6-9-D; $P < 0.001$). Genotype sp5 showed the next highest sap flow rate (0.656 µl s^{-1}) but this was statistically similar to the wild type and $59F_4$(*Ds*+) plants.

6.2.3.6 Plant ABA Contents

ABA contents in different tomato genotypes were quantified in xylem sap and in bulk leaf tissue (detail in chapter-2) by using GC-MS-MS. The xylem sap was collected 28, 42 and 56 DAG, whereas bulk leaf ABA was collected 14, 28 and 42 DAG. Not enough xylem sap could be collected in 14-day old plants, leaf samples collected from plants at 56 DAG degraded during processing.

6.2.3.6.1 Xylem sap ABA concentration [ABA_{xyl}]

The highest [ABA_{xyl}] (111.6 nM) was recorded in genotype $102F_5$(*Ds*+) at 28 DAG which was more than six fold higher compared to genotype $102F_5$ (*Ds*-) plants (18.0 nM; Figure 6-10a). This was followed by genotype sp5 plants with [ABA_{xyl}] of 89.1 nM, statistically it was similar to $59F_4$(*Ds*+) plants, but higher than $59F_4$(*Ds*-) and $102F_5$(*Ds*-). The genotype $59F_4$(*Ds*+) had similar [ABA_{xyl}] compared to the plants of $59F_4$(*Ds*-) with 54.3 nM and 36.0 nM, respectively, 28 DAG.

At 42 DAG maximum [ABA_{xyl}] (136.1nM) was recorded in genotype $102F_5$(*Ds*+) plants. This was more than three-fold higher than the genotype $102F_5$(*Ds*-) (43.7nM) and it was significantly different from other genotypes used in the experiment. However, no differences were found amongst the genotype $59F_4$(*Ds*+) and $59F_4$(*Ds*-) for [ABA_{xyl}] at 42 DAG. Genotype sp5 had significantly lower [ABA_{xyl}] (64.4 nM) compared to $102F_5$(*Ds*+) 42 DAG, however, it was statistically similar to the wild type and $59F_4$(*Ds*+) plants at this stage.

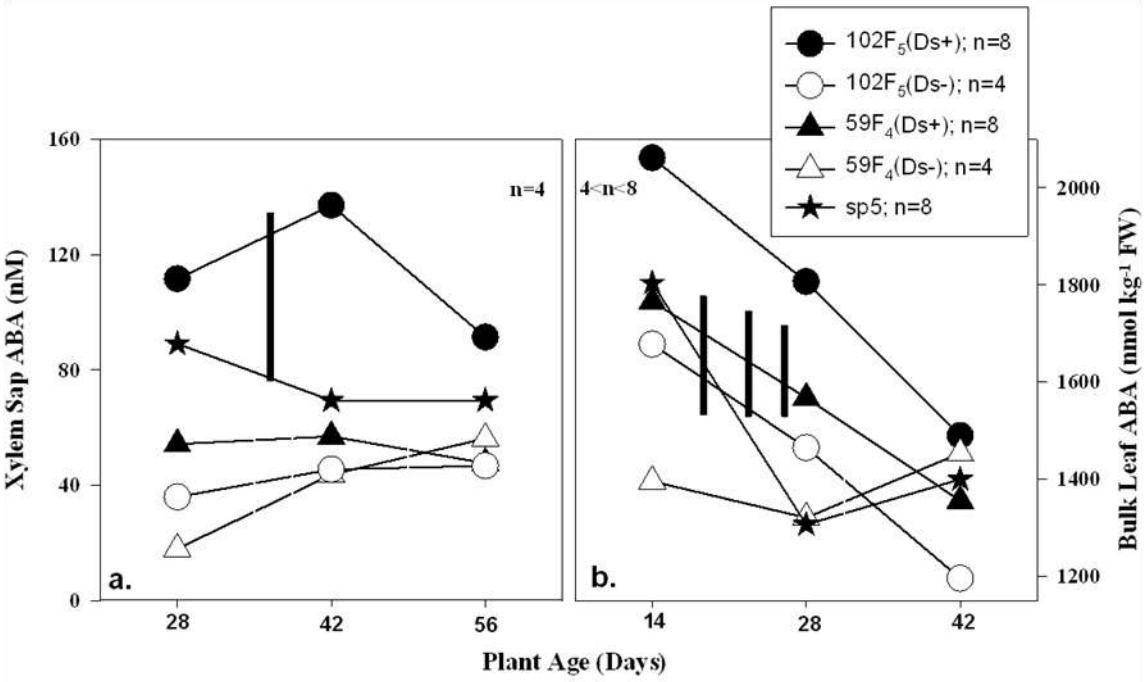

Figure 6.10. Abscisic Acid contents of leaves and xylem sap collected at different stages of plant growth. **a**. The concentration of ABA present in the xylem sap (nM), **b.** The ABA contents in leaf tissues harvested at various growth stages. Floating bars indicate *LSD* values at 5% probability level for comparing any two means, for xylem sap n=4, whereas, for Leaf bulk ABA, $4 \leq n \leq 8$, hence, one LSD bar and three *LSD* bars in case of leaf bulk ABA. Genotype sp5 was used as control, whereas $102F_5$(*Ds*-) and $59F_4$(*Ds*-) genotypes were derived from $102F_5$(*Ds*+) and $59F_4$(*Ds*+) plants, respectively, as these genotypes lost the *Ds* element through segregation.

The $[ABA_{xyl}]$ in genotype $102F_5$(*Ds*+) declined to 56 DAG, however, the highest $[ABA_{xyl}]$ (91.4nM) was still recorded in $102F_5$(*Ds*+) which was significantly higher than all other genotypes at $P < 0.05$. The next highest was sp5 (69.4 nM). Statistically there was no difference in the genotypes $59F_4$(*Ds*+) and $59F_4$(*Ds*-) with the $[ABA_{xyl}]$ concentration of 56.0 nM and 45.5 nM, respectively at 56 DAG (Figure 6-10.a).

6.2.3.6.2 Bulk Leaf ABA contents [ABA_{bl}]

Statistically the [ABA_{bl}] in genotypes containing the *Ds* element was higher than the genotypes that had lost the *Ds* element through segregation at 14 DAG ($P<0.05$). Maximum [ABA_{bl}] (2062 nmol kg^{-1} FW) at 14 DAG was recorded in genotype $102F_5$(*Ds*+) higher than $102F_5$(*Ds*-) at $P<0.001$, also the [ABA_{bl}] in genotype $59F_4$(*Ds*+) (1766 nmol kg^{-1}FW) was higher than $59F_4$(*Ds*-) plants (1678 nmol kg^{-1} FW); Figure 6-10.b.

At 28 DAG the maximum [ABA_{bl}] was in genotype $102F_5$(*Ds*+) (1807 nmol kg^{-1} FW) and this was significantly higher than all other genotypes used in the experiment ($P < 0.05$). Genotype $59F_4$(*Ds*+) had statistically higher [ABA_{bl}] compared to $59F_4$(*Ds*-) plants, with 1568 and 1321 nmol kg^{-1} FW, respectively (Figure 6-10.b).

The highest [ABA_{bl}] (1490 nmol kg^{-1} FW) was recorded in genotype $102F_5$(*Ds*+) at 42 DAG. Statistically, it was higher than $102F_5$(*Ds*-) plants, but similar to all other genotypes ($P<0.05$). The results also revealed that $59F_4$(*Ds*+) plants had statistically similar [ABA_{bl}] contents to $59F_4$(*Ds*-) at 42 DAG. No differences could be observed in [ABA_{bl}] contents of sp5 and other genotypes used during the experiment 42 DAG.

6.2.3.7 *LeNCED1* expression in the genotypes at different harvest times

The *LeNCED1* expression analysis was carried out at four different harvest stages (i.e. 14, 28, 42 and 56 DAG) using SYBR Green assay. Due to similar expression level of *LeNCED1* genotypes $59F_4$ (*Ds*-) and $102F_5$(*Ds*-). Data has been pooled and referred to as wild type in this section.

6.2.3.7.1 Genotype $102F_5$(*Ds*+)

The *LeNCED1* expression in genotype $102F_5$ (*Ds*+) was 1.5 times higher than the wild type plants 14 DAG (Figure 6-11). The *LeNCED1* expression level increased significantly and was almost nine times higher 28 DAG. The *LeNCED1* expression of $102F_5$ (*Ds*+) decreased between 28 and 42 DAG to approximately to 2.5 times more ($P<0.01$) than the wild type and remained on the same level until final harvest at 56 DAG.

6.2.3.7.2 Genotype $59F_4$(*Ds*+)

Results in Figure 6-11 show that statistically there was no difference between wild type and $59F_4$(*Ds*+) plants throughout the growth period.

6.2.3.7.3 *LeNCED1* expression in sp5

The sp5 plants had almost 3.0 and 2.5 times higher ($P<0.001$) expression of *LeNCED1* at 14 and 28 DAG, respectively, but the *LeNCED1* expression levels of both sp5 and wild type plants were similar at 42 DAG (Figure 6-11).

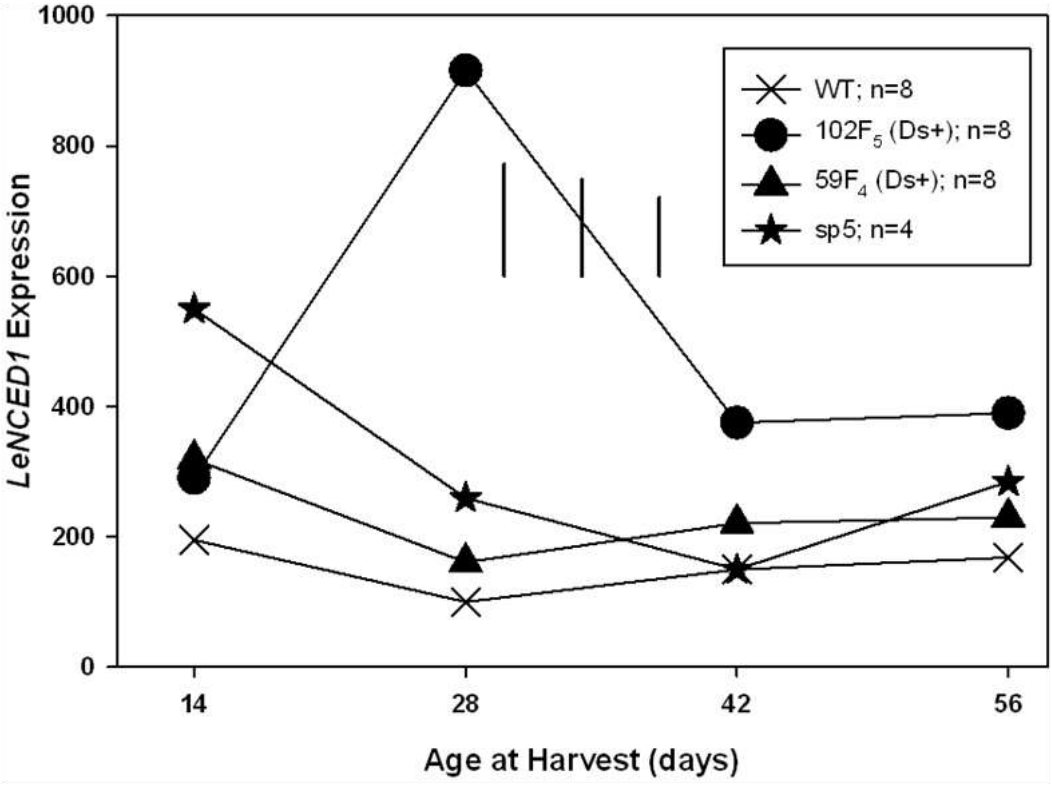

Figure 6.11. Expression analysis of *LeNCED1* gene at various harvest times in the genotypes. Expression analysis was carried out by using primers specifically made for rt-PCR, annealing in the open reading frame of *LeNCED*1 gene. Floating bars indicate the *LSD* values at $p<0.05$, three bars indicate the varying number of replicates in each block.

6.2.3.8 Conclusion

In brief, the genotype $102F_5$ (*Ds*+) had slower rate of establishment compared to genotype $102F_5$ (*Ds*-), hence a reduction in total leaf area and total plant DW. However, plants belonging to genotype $102F_5$ (*Ds*+) had reduced epinasty and Ψ_l but higher Ψ_P. This genotype also had very high [ABA_{xyl}] at 14 and 28 DAG.

The genotype $59F_4$(*Ds*+) had similar growth rate and biomass accumulation compared to $59F_4$(*Ds*-) at various harvest stages. Similarly, no differences could be detected between the genotypes for plant water relations except Ψ_P which was more in

$59F_4$(*Ds*+) plants compared to $59F_4$(*Ds*+) plants. No differences in the xylem sap ABA could be recorded between the two genotypes, however, [ABA_{bl}] were higher in the genotype $59F_4$(*Ds*+) at 14 and 28 DAG when compared to $59F_4$(*Ds*-).

The sp5 plants had slower establishment rate compared to the wild type plants, although no suitable control was used to evaluate this genotype correctly. This genotype had initially lower LA and plant DW but at the time of final harvest (i.e. 56 DAG). These plants had higher LA and DW compared to all other genotypes used in the experiment. Further, these plants had lower Ψ_l and high Ψ_p with high [ABA_{xyl}] at 28 DAG.

6.3 Correlation between ABA and plant growth traits

Correlation between [ABA_{bl}] and [ABA_{xyl}] was plotted against above ground plant growth at various harvest stages, this has been described below.

6.3.1 Correlation between [ABA_{bl}], leaf area and dry weight

The [ABA_{bl}] was plotted against LA (Figure 6-12.a) for 14 day old plants, and a strong negative correlation between the two variables was observed (r^2 = -0.44). Similarly, the correlation between the [ABA_{bl}] and total plant DW (Figure 6-12.b) was found to be negative (r^2 = -0.39), suggesting that [ABA_{bl}] reduced LA and biomass accumulation at 14 DAG. A weak negative correlation (r^2 = -0.15) shown in Figure 6-12.c was observed at 28 DAG. Similarly, the [ABA_{bl}] and plant DW were found to have a weak negative correlation (r^2= -0.12), shown in Figure 6-12.d, however, these correlations were statistically not significant at $P<0.05$. Further, a negative, weak and non-significant correlation (r^2= -0.11) was found between plant LA and [ABA_{bl}] (Figure

6-12.e) at 42 DAG. There was no correlation between plant DW and [ABA_{bl}] at 42 DAG (Figure 6-12.f).

6.3.2 Correlation between [ABA_{xyl}] and vegetative growth parameters in the genotypes

As described earlier, xylem sap could not be collected from 14 days old plants, only ABA contents could be quantified in 28, 42 and 56 days old plants. [ABA_{xyl}] was plotted against LA and DW to find a correlation, if any, between these parameters (Figure 6-13).

A negative correlation between [ABA_{xyl}] and LA (r^2 = -0.22) was observed in plants 28 DAG (Figure 6-13.a). Similarly, a weak negative correlation was found between ABA and DW, however, in both cases there the correlation was not significant. ($P > 0.05$) (Figure 6-13 a & b). A similar correlation pattern was observed between [ABA_{xyl}] and both LA and DW at 42 DAG, but at this stage it became significant ($P < 0.05$) (Figure 6-13 c & d) suggesting that LA and DW were reduced due to higher ABA concentration in the xylem sap. A similar trend was observed at 56 DAG but the correlation at this time point was weaker and non-significant (Figure 6-13 e & f).

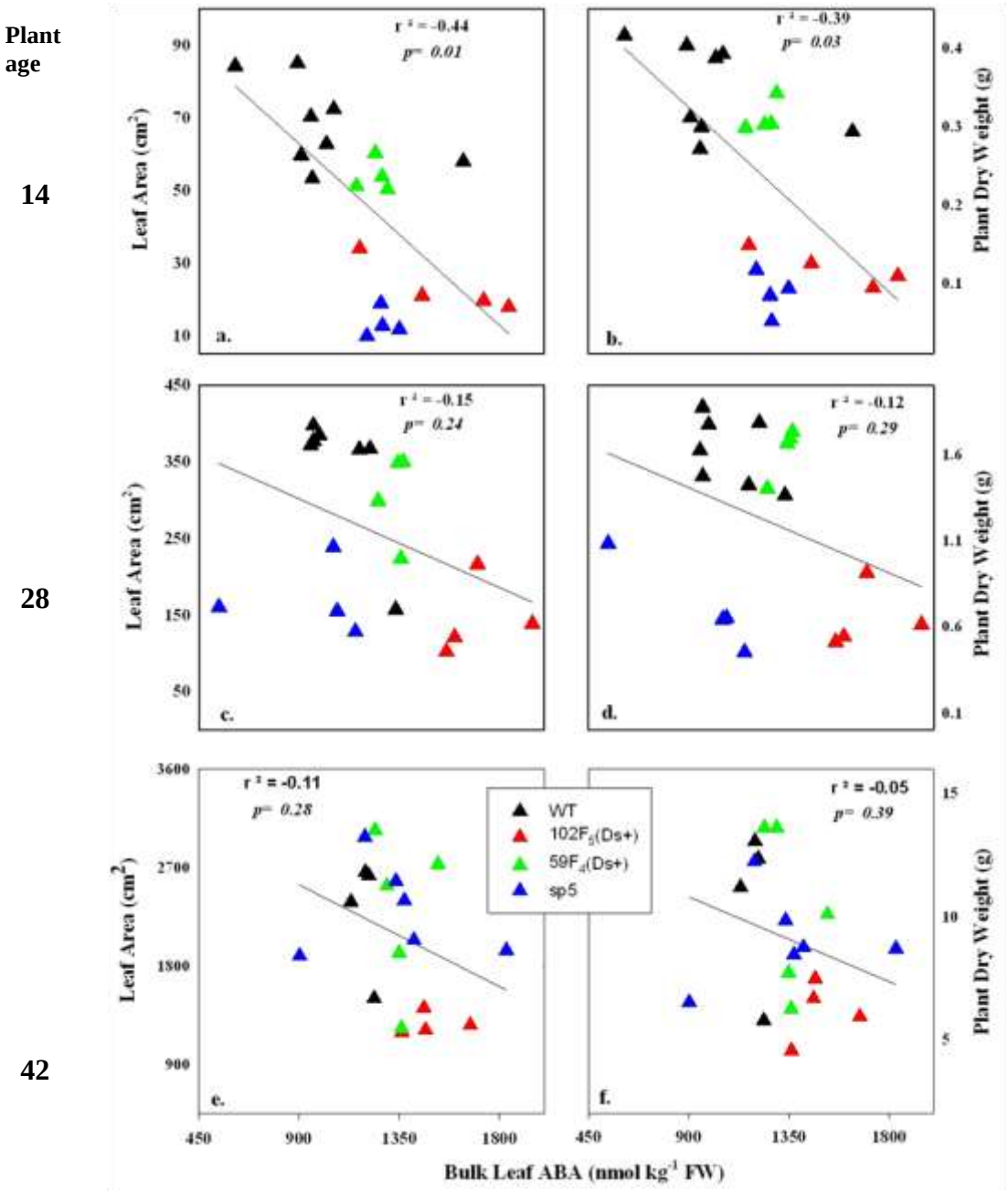

Figure 6.12. Correlation between ABA concentration in the leaf tissue, leaf area and shoot dry weight in different genotypes at various time intervals. Panel 'a' and 'b' shows the correlation in 14 days old plants, 'c' and 'd' in 28 days old plants and 'e' and 'f' in 42 days old plants. Genotypes $102F_5$(*Ds*+) and $59F_4$(*Ds*+) plants retained the *Ds* elements while $59F_4$(*Ds*-) and $102F_5$(*Ds*-) lost the *Ds* element through segregation termed as wild type in this figure, data from both wild type was pooled. Where, 'r^2' indicates the coefficient of correlation and $n \leq 4 \leq 8$. , p-value was calculated from a critical table for Pearson's correlation coefficient.

6.4 Gravimetric Analysis of plant Water used in Genotypes $59F_4$(*Ds*+) and $102F_5$(*Ds*+)

6.4.1 Plant Dry Biomass Produced (g)

Total biomass produced by individual plants in the experiment was adjusted through the subtraction of starting DW of each genotype. Although there were statistically significant differences in initial DW among different genotypes, the plants used in the gravimetric experiment were selected on the basis of visually similar size.

The plants were harvested just above the ground level and dried to a constant weight. Results showed that there were significant differences in genotypes ($P<0.05$) for DW at the end of gravimetric experiment, which lasted for approximately 3-weeks. Maximum DW was produced by sp5 plants (45.08 g). This was 15 and 27% percent higher than $59F_4$(*Ds*-) and $102F_5$(*Ds*-) plants, which produced 38.36 g and 33.03 g, respectively. The genotype $102F_5$(*Ds*+) produced the lowest DW (26.20 g). This was approximately 21% lower than $102F_5$(*Ds*-) plants and 40% lower than sp5 plants, and was significantly lower than all other genotypes at $P<0.001$ used in the experiment (Table 6-4).

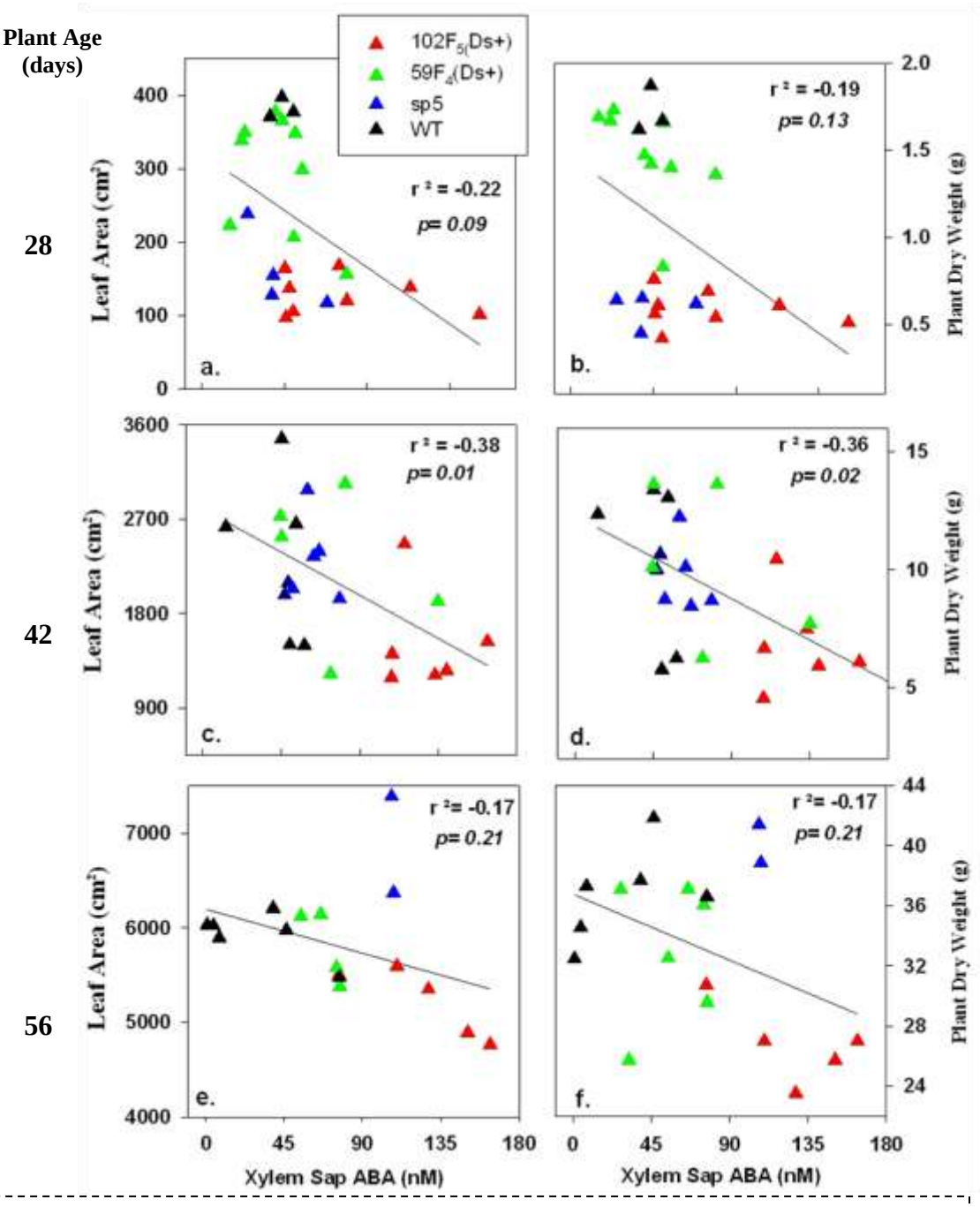

Figure 6.13. Correlation between ABA concentration in the xylem sap, leaf area and shoot dry weight in different genotypes at various time intervals. Panel 'a' and 'b' shows the correlation in 28 days old plants, 'c' and 'd' in 42 days old plants and 'e' and 'f' in 56 days old plants. Genotypes $102F_5$(*Ds*+) and $59F_4$(*Ds*+) retained the *Ds* elements while $59F_4$(*Ds*-) and $102F_5$(*Ds*-) lost the *Ds* element through segregation, termed as wild type in this figure, data from both wild type was pooled. Where, 'r^2' indicates the coefficient of correlation and $4 \leq n \leq 8$. , *p*-value was calculated from a critical table for Pearson's correlation coefficient. Note at different stages some plants did not produce any sap, especially the wild type plant, hence, fewer points on such figures.

6.4.2 Total Plant Transpiration (kg H_2O)

Total water lost through transpiration was recorded over the period of the gravimetric experiment (Chapter-2). Genotype $102F_5$ (*Ds*-) transpired significantly more water (7.89 kg H_2O) compared to the genotype $102F_5$ (*Ds*+) with water loss of only 3.63 Kg, (54% less transpiration). This was significant at $P<0.001$. The transpiration in genotype $59F_4$(*Ds*+) (6.97 kg H_2O) was 10.8% lower than the $59F_4$(*Ds*-) plants which transpired 7.58 kg H_2O, but statistically there was no difference in the two genotypes (Table 6-4).

Results in Table 6-4 also showed that the sp5 plants transpired 7.18 Kg H_2O. This was nearly 50% more than genotype $102F_5$ (*Ds*+) and significantly higher at $P<0.001$. However, the total transpiration in sp5 plants was statistically similar to $102F_5$ (*Ds*-), $59F_4$(*Ds*+) and $59F_4$(*Ds*-) plants (Table 6-4).

6.4.3 Plant Water Use Efficiency (g DW kg^{-1} H_2O)

There were significant differences between the genotypes in the WUE_p (Table 6-4) at $P<0.05$. The WUE_p (7.22 g DW kg^{-1} H_2O) was highest in genotype $102F_5$(*Ds*+) plants. This was significantly higher than all other genotypes used in this experiment ($P<0.001$) and 33% higher than WUE_p of $102F_5$ (*Ds*-) plants. This was followed by genotype sp5 (6.28 g DW $kg^{-1}H_2O$) which had 13% lower WUE_p compared to genotype $102F_5$ (*Ds*+) plants. The genotype $59F_4$ (*Ds*+) did not show any significant differences from genotype $59F_4$ (*Ds*-) or genotype $102F_5$ (*Ds*-) plants, with only 9% more WUE_p than its wild type i.e. $59F_4$ (*Ds*-) plants, while genotype $59F_4$ (*Ds*-) proved to have the lowest WUE_p (42% lower) compared to $102F_5$ (*Ds*+) (Table 6-4).

6.4.3.1 Correlation between WUE_p and Phenotypic traits in genotype $59F_4$ (*Ds+*) and $102F_5$ (*Ds+*) plants

A comparison was made between different phenotypic traits and WUE_p in 56 day old plants. The results are present in Figure 6-14.

6.4.3.1.1 WUE_p vs Dry Biomass gained

The results across all the genotypes used during the experiment revealed a weak negative correlation (r^2= -0.11) between WUE_p and total dry biomass produced by 56 day old plants, and not statistically significant at *P<0.05* (Figure 6-14-a).

6.4.3.1.2 WUE_p vs Petiole length

Previously it was reported (Thompson et al., 2007) that plants with higher WUE_p had higher petiole length. The results in this experiment revealed a strong positive correlation between the two parameters (r^2= 0.38; *P<0.001*), confirming the previous results that the plants with higher WUE_p had longer petioles (Figure 6-14-b).

6.4.3.1.3 WUE_p vs Leaf Area

The results from WUE_p and LA in 56 day old plants (Figure 6-14-c) revealed that there was no correlation between the two variables (r^2= 0.04).

6.4.3.1.4 WUEp vs [ABA_{xyl}]

Higher [ABA_{xyl}] contents increased WUE_p as indicated by a strong positive correlation between the two variables (r^2= 0.65; *P<0.001*) shown in Figure 6-14-d.

6.4.3.1.5 WUEp vs time to flowering

The comparison between the two variables (Figure 6-14-e; *P<0.001*) revealed a positive correlation (r^2= 0.33).These results revealed that the plants with higher WUE_p took more time to reach flowering stage.

6.4.3.1.6 WUE_p vs *LeNCED1* expression

A strong positive correlation (r^2= 0.67; *P<0.001*) was observed between WUE_p and *LeNCED1* expression determined at the time of final harvest at 56 DAG, (Figure 6-14-f).

Table 6-4: Effect of *LeNCED1* overexpression on plant water use efficiency in genotypes $59F_4$ and $102F_5$.

	Genotypes						
Replications	12	4	12	4	8		**ANOVA**
	$102F_5$(*Ds*+)	$102F_5$(*Ds*-)	$59F_4$(*Ds*+)	$59F_4$(*Ds*-)	sp5	*p value*	*LSD*
Initial biomass (g dry wild type)	6.19[b]	9.46[a]	10.98[a]	11.29[a]	9.45[a]	0.002	3.01 min 2.651 max-min 2.164 max
Biomass produced (g dry wild type)	26.20[c]	38.36[b]	33.26[b]	33.03[b]	45.08[a]	<0.001	5.505 min 4.495 max-min 3.178 max
Transpiration (Kg H_2O)	3.63[c]	7.89[a]	6.97[b]	7.58[ab]	7.180[ab]	<0.001	0.917 min 0.749 max-min 0.529 max
WUE_p (g dry wild type kg H_2O^{-1})	7.22[a]	4.86[c]	4.77[c]	4.36[c]	6.28[b]	<0.001	0.709 min 0.579 max-min 0.409 max

Note: Different letters in the same row indicate statistically significant differences in a variate *at LSD* 5% or less.

$102F_5$(*Ds*+) and $59F_4$(*Ds*+); genotyping contained the *Ds* element; whereas, $102F_5$(*Ds*-) and $59F_4$(*Ds*-) ; lost the *Ds* element through segregation. Plant transpiration was measured by using gravimetric method over a period of 23 days. Only aboveground biomass was determined over the same period. Initial biomass was determined at the beginning of this experiment. P values standard error of difference in means (SED) and LSD (least significant difference) were obtained from ANOVA (5%).

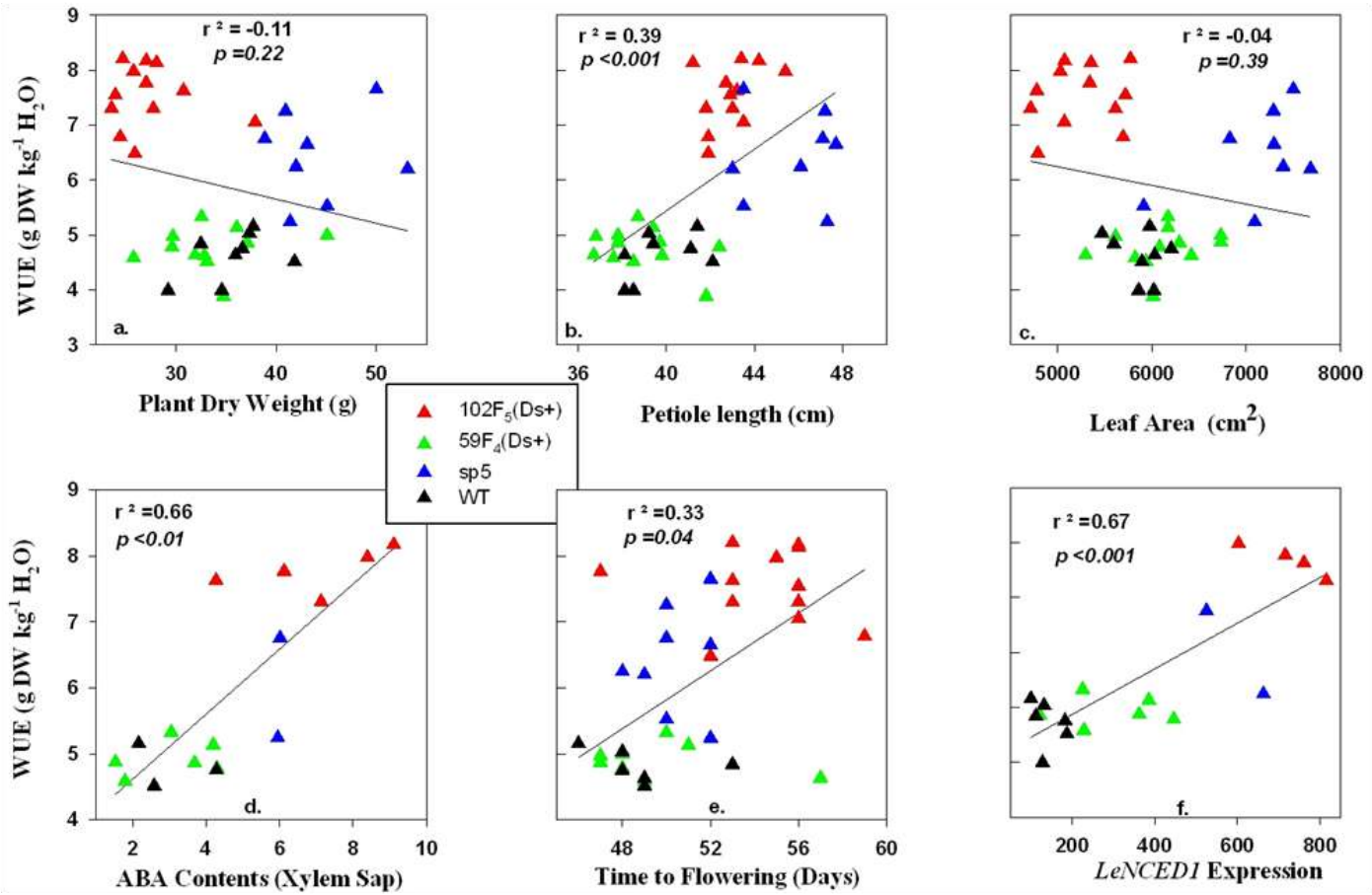

Figure 6-14. Correlation between WUE_p and various growth and physiological parameters observed in different genotypes. Panel 'a' shows correlation between WUE_p and plant DW (g); b, correlation between WUE_p and Petiole length (cm); c, correlation between WUE_p and LA (cm^2); d, correlation between WUE_p and ABA contents in xylem sap; e, correlation between WUE_p and time required to reach flowering and f, correlation between WUE_p and *LeNCED1* gene expression. 'r^2' shows a coefficient of correlation, *p-value* was calculated from a critical table for Pearson's correlation coefficient.

6.5 Correlation between ABA_{xyl} and various plant features

Data obtained from differernt plant growth parameters was plotted against [ABA_{xyl}]. The plants with higher [ABA_{xyl}] had longer petioles (Figure 6-15.a) indicated by a strong positive correlation (r^2 = 0.51; *P=0.01*). Leaf epinasty had a negative correlation (r^2 = -0.40; *P* = 0.02) suggesting that higher [ABA_{xyl}] reduced the extent of epinasty (Figure 6-15.b). The correlation between [ABA_{xyl}] and time to start flowering was positive (r^2= 0.34; *P=0.04*), implying that higher [ABA_{xyl}] delayed flowering in plants (Figure 6-15.c) and a strong positive correlation (r^2= 0.70; *P<0.001*) between [ABA_{xyl}] and *LeNCED1* expression confirmed the expected causal relationship between NCED expression and ABA accumulation suggested that high gene expresssion was associated with high [ABA_{xyl}].

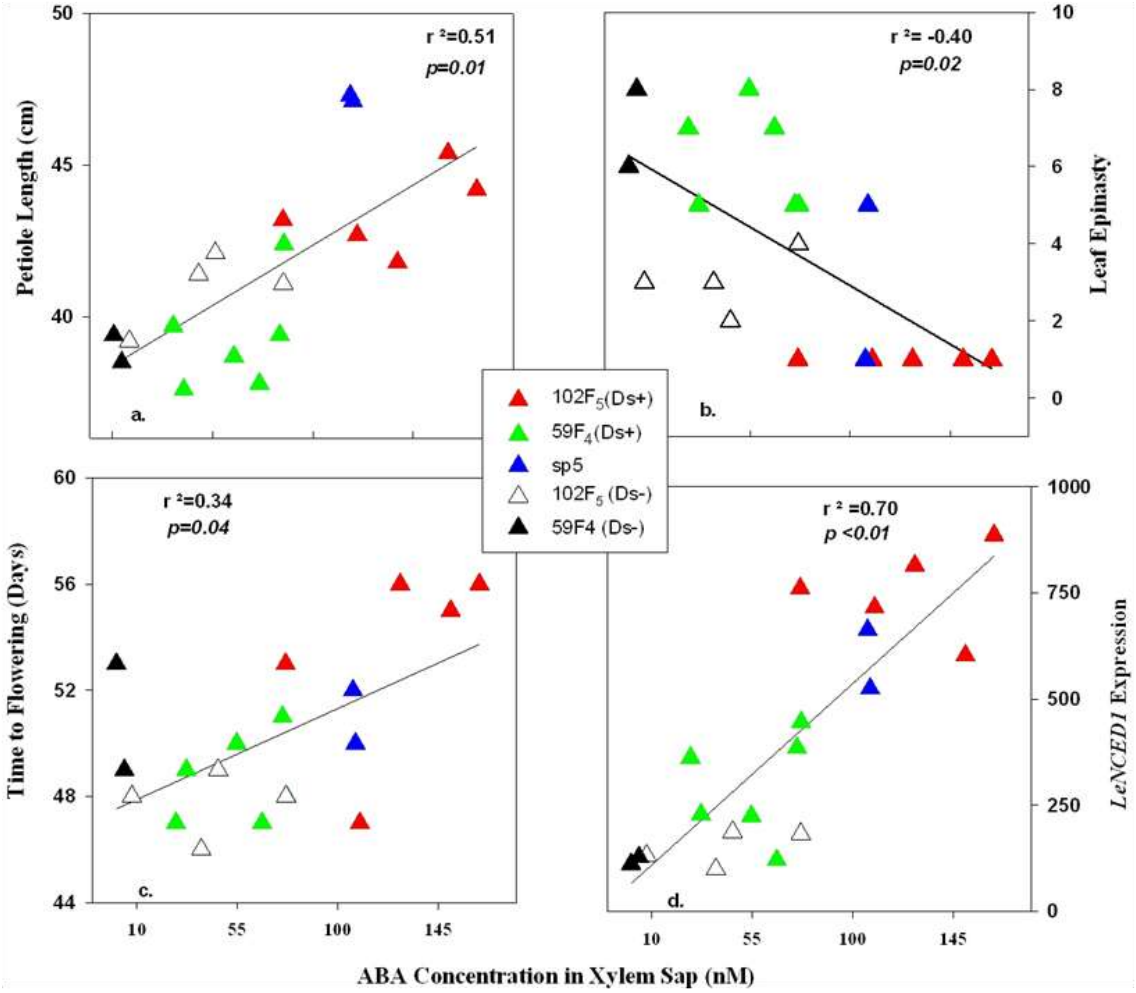

Figure 6-15. Correlation between ABA concentrations in the xylem sap and various plant parameters in different genotypes of 56-days old tomato plants. Correlation between xylem sap ABA and a, petiole length (cm); b, Leaf epinasty; c, time to start flowering; d, *LeNCED1* expression. Genotypes $102F_5$ (*Ds*+) and $59F_4$ (*Ds*+) retained the *Ds* elements while $59F_4$ (*Ds*-) and $102F_5$ (*Ds*-) lost the *Ds* element through segregation. Where, 'r^2' indicates the coefficient of correlation and *p-value* was calculated from a critical table for Pearson's correlation coefficient.

6.6 Discussion

Genotypes $59F_3$ and $102F_3$ exhibited symptoms of putatively high ABA, such as reduced g_s (chapter-5 section 5.3.2.4 and 5.3.3.4), grey leaf colour and visually higher leaf angles as previously observed by Thompson *et al*. (2000). In order to validate that these genotypes had such symptoms due to overexpression of the *LeNCED1* gene transposed at various positions in the genome, these genotypes were assessed for their establishment, relative growth rate (RGR), net assimilation rate and total above ground dry matter contents at various growth stages. Xylem sap and bulk leaf ABA contents and *LeNCED1* expression were also measured various growth stages, and the gravimetric plant water use was measured in order to calculate the plant water use efficiency (WUE_p).

6.6.1 Effect of ABA on plant growth:

There has been conflicting evidence on the role of ABA as a growth hormone with some studies suggesting ABA as a growth inhibitor (Rehm and Cline, 1973, Cramer and Quarrie, 2002, Tung et al., 2008) and other studies suggesting that at selective concentrations. For example, ABA acts as a growth promoter in duck weed and increases the plant fresh weight (McWha and Jackson, 1976). However, most of this previous work concentrated on the inhibitory effects of ABA; until, Sharp et al. (2000) and Thompson et al. (2007a) reported that ABA does not always act as a growth inhibitor and can help plant tissues expansion.

6.6.1.1 Correlation of plant growth with [ABA_{xyl}]

On the basis of [ABA_{xyl}], the genotypes used during this study can be divided into three groups: A, *High ABA genotype* ($102F_5$(*Ds*+) ;B, *Medium ABA Genotype* (sp5); and C, *Wild type ABA Genotype* which also includes $59F_4$(*Ds*+) and was not significantly different from the wild type in [ABA_{xyl}].

It is assumed that the *LeNCED1* gene driven by HistoneH2A promoter in the genotype $102F_5$(*Ds*+) had integrated in the plant genome near a promoter region which influenced the production of ultra high ABA in this genotype resulting in slow plant growth and lower DW accumulation. In a similar study the overexpression of *LeNCED1* by using Rubisco, a highly active promoter in the photosynthetic tissues (Gittins et al., 2000), in tomato genome resulted in slow plant growth and establishment and DW accumulation (Tung et al., 2008).

As described above, ABA has been found to be an important phytohormone which helps in maintaining a positive cell Ψ_p necessary for leaf expansion (Thompson et al., 2007). In *high* and *medium* ABA genotypes lower g_s results in lower water losses and higher Ψ_1 this can give rise to high Ψ_p at similar Ψ_s as observed in section 6.2.3.5.2, Figure 6.9. This has been observed in the sp5 line which probably contains 'optimum ABA' concentration in the xylem sap necessary for rapid growth and higher DW accumulation. This optimum [ABA_{xyl}] might have resulted in maximum possible reduction in plant g_s without affecting average plant growth rate or rate assimilation (Ortega, 2010, Thompson et al., 2007).

However, the *Wild type* or 'sub-optimal' [ABA_{xyl}] cannot reduce the g_s, hence more water loss through transpiration (Tardieu et al., 1996) as observed in the wild type

plants. Under such circumstances, the cell Ψ_p cannot be maintained which is required for growth and DW accumulation.

Genotype $59F_4$(*Ds*+) which consistently showed lower g_s in the F_3 generation (Chapter-5, section 5.3.3.4) had similar biomass accumulation as wild type plants with no differences in WUE_p in F_4 plants (section 6.4.3). One of the most plausible reasons might be the silencing of the endogenous NCED gene in F_4 progeny. This can be due to inactivation of the *Ds* element/*Tr-Ds* element on a particular locus through production of antisense transcripts (Greco et al., 2003). It might also be due to the loss of a specific *Ds* element by segregation which could not be detected by a mere PCR, as the PCR can only detect the presence or the absence of a gene and it cannot reveal the segregation of genes at various loci.

6.6.2 Phase specific role of ABA in different genotypes

Plant architecture, its physiology and tissue sensitivity to phytohormones changes throughout plant ontogeny and responds to environment as well as resource availability (Evans and Poethig, 1995). Changes in hormonal concentration are of key importance at various growth stages which could modify the plant morphological traits. Higher NAR (section 6.2.3.3.4) and RGR (section 6.2.3.3.5**)** in $102F_5$(*Ds*+) and sp5 during later growth period (28-56 DAG) may indicate that ABA was responsible for improved growth. Perhaps the older plants were not sensitive to the growth inhibitory effect of higher concentration of ABA. The increased RGR and NAR in the genotype $102F_5$(*Ds*+) and sp5 might be related to an alteration of resource (photosynthates) allocation to photosynthetic organs such as leaves.

Similarly, the results of correlation between LA and DW with [ABA_{bl}] at different growth stages suggest that there might be a negative interaction between the two variables. Because as the plant tissue grew older the sensitivity of these tissues to [ABA_{bl}] decreased (section 6.2.3.6.1, Figure 6-12) indicated by stronger correlation between ABA and growth traits at younger age but weak or no correlation between the tissue ABA and the growth traits at later stages. This is in line with the results obtained by (Atkinson et al., 1989); they demonstrated that the younger leaves were more responsive to exogenous application of ABA and as leaves aged they became less sensitive to its application indicated by a rapid decrease in the g_s of younger leaves in comparison to the older leaves.

However, previously it was found that [ABA_{xyl}] showed a better correlation with plant growth, development and stomatal behaviour as compared to the [ABA_{bl}] contents (Tardieu et al., 1992, Khalil and Grace, 1993). This is not entirely in agreement with the findings in this study, which suggests that the plants might have been more sensitive to [ABA_{xyl}] at 42 DAG only (Figure 6-13). This might be due to the xylem ABA concentration which strongly influences the perception of ABA by receptors, and the main ABA receptors are interacellular (Ma et al., 2009).

6.6.3 Role of ABA in convalescence of plant water relations

The ability of plants over-expressing *LeNCED1* to maintain high Ψ_l has key advantage over wild type plants and maintenance of Ψ_l under drier environments has even more significance as the plants can maintain a normal or near normal growth rate. Another key difference between plants grown under drought and well watered conditions is the ability of transgenic plants ($102F_5$(*Ds*+) and sp5) to conserve water in

the root zone for a longer period of time due to reduced g_s as a result of higher [ABA], hence use less water even under well watered conditions. Whereas, the wild type plants are only able to reduce g_s when the roots sense the drier rhizosphere (Blackman and Davies, 1985, Zhang and Davies, 1989, Meyer and Gingrich, 1964) or if Ψ_l has decreased to -1.0 MPa or below in *Ambrosia trifida* L and *Ambrosia artemisiifolia L*(Zabadal, 1974).

The observation that genotype $102F_5$(*Ds*+) guttated (Chapter-5, section 5.3.2.2) at 65% RH, a relatively low VPD at which sp5 did not guttate (Thompson et al., 2000), and showed a greatly increased rate of sap flow from detopped plants (up to 4.0 ml hr^{-1}) meant that these plants might have higher Lp_r, as found by (Thompson et al., 2007) or simply that to the plants had a higher root biomass (root biomass was not measured). This was accompanied by lower transpiration rate maintaining higher Ψ_p in genotype $102F_5$(*Ds*+). While, no guttation was observed in sp5 plants at 65% RH, further the sap flow rates up to 2.0 ml hr^{-1} observed in sp5 were not as high as recorded in $102F_5$(*Ds*+) (section 6.2.3.5.3), although this is not conclusive as the sap flow rate is dependent on the size of the root system. Previously, it was observed that a higher Lp_r could be achieved by application of exogenous ABA (Hose et al., 2000). In the opposite scenario the inability of ABA deficient mutant *vp14* to maintain high Ψ_P can be a result of low Lp_r (Tal and Nevo, 1973, Parent et al., 2009).

As previously mentioned, ABA causes a buildup of Ψ_p by increasing the water flow through activation of aquaporins (Wan et al., 2004) and inhibition of cell wall loosening as found by Gimeno-Gilles *et al.* (2009) during their work on Medicago which resulted in increased Ψ_p. Its noteworthy here that the increase in the Ψ_p can also be due to a reduced g_s, which is consistent even under well watered conditions. The

presence of brittle plant tissues in these high ABA genotypes ($102F_5$(*Ds*+ and sp5, an observation only) suggests that there was little capacity of the tissue to bend and that this was mediated by high Ψ_p and low cell wall extensibility. This could help maintain growth in plants, restoring Ψ_l which might not be achieved in wild type plants transpiring at much higher rates.

In the present study, it was observed that in genotype $59F_4$(*Ds*+), the morphological traits (i.e. rate of LA expansion, biomass accumulation, RGR and NAR) were similar to the wild type plants. This is due to similar amount of ABA in the leaf and the xylem sap during most of the growth phases. In genotype $102F_5$(*Ds*-) there was higher DW accumulation compared to $59F_4$(*Ds*+) and $59F_4$(*Ds*-) plants which can be supported by the absence of leaf epinasty (section 6.2.3.4.3).

6.6.4 Antagonistic relationship between [ABA_{xyl}] and leaf epinasty

Reduced ABA level in the wilty tomato mutant *flacca* is responsible for its abnormal morphological features (Tal et al., 1979) where leaves fail to expand and also curl up to 360^o due to unequal tissue expansion. The reduced epinasty in $102F_5$(*Ds*+) and sp5 plants is in agreement with the results obtained in one of the studies by LeNoble *et al*. (2004), who found that the effect of epinasty was eliminated by exogenous application of ABA on deficient mutants in *A.thaliana*, suggesting an antagonistic relationship between ABA and ethylene (LeNoble et al., 2004) which acts as a growth inhibitor. Leaf epinasty causes leaves curling and twisting (Figure 6-8), reducing the net photosynthetic area as the curled up leaves are unable to utilize the natural resources such as light and aeration for efficient photosynthesis during the day.

The observation that genotype $102F_5$(*Ds*-) did not have any sign of leaf epinasty even though its ABA levels were lower, compared to $59F_4$(*Ds*+) and $59F_4$(*Ds*-) plants, suggested that the genotype $59F_4$(*Ds*+) might have been more sensitive to the presence of ethylene (data on ethylene production not recorded). Alternatively this might be due to some other genetic mutation independent of the *Ds* element as both genotypes ($59F_4$(*Ds*+) and $59F_4$(*Ds*-) showed leaf epinasty (section 6.2.3.4.3). Higher extent of leaf epinasty in these genotypes can also be attributed to a mixed genetic background as the parent plant containing the *sAc* and *Ds element* were from Money Maker and Ailsa Craig background, respectively.

6.6.5 The [ABA_{xyl}] and time to reach reproductive maturity:

Plant transition to floral organ development is an integrated response to various internal and external environments. As previously mentioned, many studies have found ABA as a growth inhibitor and it was found that significant accumulation of ABA at the growing regions of plants inhibited floral initiation (Sharp et al., 2009). However, (Hall and McWha (1981) showed that the exogenous application of ABA promoted flowering in a wheat crop (Hall and McWha, 1981).

In the present study, genotype sp5 did not show signs of delayed flowering even though it had higher [ABA_{xyl}] than the wild type plants. However, the flowering was delayed in genotype $102F_5$(*Ds*+) which could be a consequence of reduced carbon assimilation as a result of low g_s (Castro et al., 2009) mediated by very high [ABA_{xyl}]. It will be interesting to know the implications of constitutively higher amounts of ABA in these transgenic plants and biomass partitioning towards the fruit set and development.

6.6.6 Plant Biomass Partitioning in different Genotypes

Plant biomass distribution between source and sink tissues allows plants to acclimate under stress conditions by altering their plant growth and development due to the presence of ABA in stressed plants (Roitsch and Gonzalez, 2004). More biomass allocation to the petiole and lamina in genotype $102F_5$(*Ds*+) and sp5 (section 6.2.3.4.1, Table 6-3) suggests that ABA has mediated the supply of soluble hexoses through phloem to the parts with higher Ψ_p for cell expansion. In a recent study, Seiler et al. (2011), showed a positive correlation between ABA in the flag leaves of barley plants and mobilization of starch contents for storage in the developing seeds. They also reported up regulation of ABA responsive *cis*-elements found in the promoter regions of important starch biosynthesis genes such as *HvSUS1* and *HvAGPL1* resulting in increase in starch biosynthesis in these plants (Seiler et al., 2011). Similar observations were reported by (Yang et al., 2000) in wheat plants.

The longer petioles in sp5 and greater leaf area with larger leaf angles suggest that these plants can ideally adapt in climates with less total sunshine hours available, as they can harvest more light energy for higher photosynthetic activity. The allocation of resources to storage tissues such as stem in genotype 59F4(*Ds*+) and wild type genotypes, meant that less resources could be allocated towards photosynthetic tissues, this might be the reason behind slower NAR, RGR and leaf expansion rate in these genotypes observed in 42 and 56 days old plants.

6.6.7 Relationship between WUE_p and DW accumulation in high ABA genotypes

The role of ABA in improving WUE_p by reducing the g_s is a controversial part of plant research. In plants with higher WUE_p a tradeoff between DW and WUE_p are undesirable because high yield and low water use is the agronomic goal (Blum, 2005). It is argued that the stomata needs to remain open with less stomatal resistance to escaping water for higher rate of carbon assimilation (Fischer et al., 1998, Condon et al., 1987) for rapid growth and development.

In all three experiments conducted during this study for gravimetric WUE_p, the results consistently showed that the $102F_5$(*Ds*+) and sp5 had higher WUE_p compared to $59F_4$(*Ds*+) and eild type genotypes (Table 6-4). But the $102F_5$(*Ds*+) produced significantly lower DW (Table 6-4) when compared to the wild type and sp5 plants. However, this would not be justifiable to compare sp5 and $102F_5$(*Ds*+) as the genetic background in both of these genotypes was different.

These results suggest optimum level of ABA present in genotype sp5 at 42-56 DAG. However, slower establishment rate of this genotype (14-28 DAG) is evident from the results in Figure 6-4, which can be due to high tissue sensitivity to ABA at this stage. A reduced carbon assimilation rate due to restricted stomatal pores mediated by very high ABA contents in genotype $102F_5$(*Ds*+) might be responsible for low final DW, suggesting that very high ABA contents will result in higher WUE_p but the DW accumulation might be greatly reduced.

Since the Southern blot shows that nearly all the selected lines have the original T-DNA band still present (chapter4; Figure 4-9, chapter-5; section 5.3.2.3 and 5.3.3.3), it was important to establish whether the non-transposed T-DNA-Ds elements were

stably integrated and inherited to the next generation and contributed to the reduction of plant g_s. An attempt was also made to remove the T-DNA element, hence, antibiotic resistance gene from genotype $102F_2$(Ds+). This is addressed in the next chapter.

Chapter-7 Effect of non-Transposed T-DNA-Ds elements on plant phenotype

This chapter describes the role of T-DNA-Ds element on plant g_s in Ds517-1 (Parent Ds-line) and its subsequent removal by backcrossing a selected genotype ($102F_2$(Ds+) with the wild type plants.

7.1 Background Information

The original crosses between the two lines (*Ac* element × T-DNA-Ds element) to activate the *Tr-Ds* element, (shown in Figure 7-1), in tomato plants were performed by Dr. Ian Taylor at Nottingham University (detailed in chapter-3) but no tissue was preserved from the individual Ds517-1 plant. Only the siblings and the progeny from selfing this parent plant was available for the study. Experiments were performed in order to determine if the original insertions of the T-DNA-Ds elements in parent Ds-line 517-1 had any effect on seed germination, leaf g_s and overall plant growth.

7.2 T-DNA-Ds elements caused seed dormancy in Ds517-1 T_2

Seeds obtained from the T_2 siblings of the parental line were germinated to determine the number and identity of the original T-DNA bands in the population through Southern blotting, and to establish if these bands influenced g_s. It was observed that the seed germination of Ds517-1 T_2 on dH_2O was very low (Figure 7-2), only 20 and 22% germination could be achieved in lines Ds517-1-1 and Ds517-1-2, respectively, 8 days after sowing; whereas, 94% of the wild type seeds germinated 8 days after imbibition. None of the germinated seeds contained the T-DNA-Ds element

and all the seedlings were wild type. Even by using of 3.3 μM norflurazon, only few seeds germinated (data not shown). To improve this, 6.50 μM norflurazon was used, but because higher doses of norflurazon enhance photobleaching due to inhibition of carotenoid biosynthesis (Bartels and Watson, 1978), seeds were germinated on agar medium (Haughn and Somerville, 1986) containing 0.8% sucrose for improved seedling establishment and better plant recovery after germination.

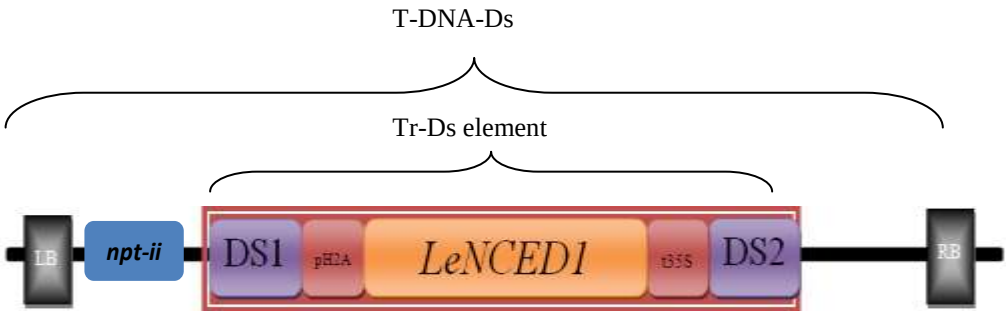

Figure 7-1. A schematic representation (not to scale) of T-DNA-Ds (nptII:Ds1:pH2A::*LeNCED1*::t35s:Ds2) and Tr-Ds (Ds1:pH2A::*LeNCED1*::t35s:Ds2) construct. LB and RB represent left and right borders, respectively. The nptII is the kanamycin resistance gene. Ds1 and Ds2 are dissociation elements; pH2A the Histone promoter; t35S is the terminator sequence from CaMV; *LeNCED1* is the tomato 9-cis-epoxy carotenoid dioxygenase gene. T-DNA-Ds was transformed through *Agrobacterium tumefaciens* transformation while *Tr-Ds* element is activated by *sAc* element.

7.3 Genotypic analysis of Ds517-1 T_2

Primer pair *Ds1For2* and *notRev5* were used to detect the T-DNA-Ds element in Ds517-1 T_2. It was observed that out of 52 plants tested 38 retained while 14 plants lost the T-DNA-Ds element. The segregation ratio (3:1) was confirmed by Chi-square test

(Table 7-1). This demonstrated that the T_2 progeny of Ds517-1 line was heterozygous for a single T-DNA locus.

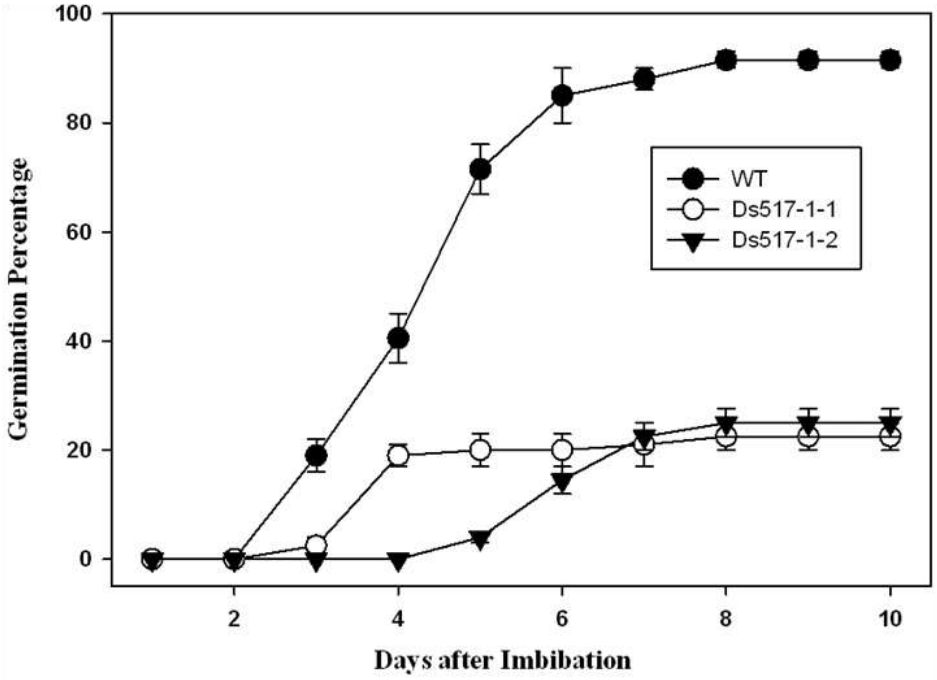

Figure 7-2. Seed germination of wild type and T_2 progeny from Ds517-1T_2 (Parent Ds line). Each data point represents the average of two Petri dishes each containing 25 seeds from each genotype were sown on filter paper soaked with dH_2O. The error bars show the standard error of means calculated from the two replications.

Table 7-1: Segregation ratios of Ds517-1 T_2 for T-DNA-Ds locus

GENOTYPE	Bands Present	No.of plants observed	No.of plants expected	x^2 calculated	x^2 0.05,1	Ratio 3:1
Ds517-1 T_2	T-DNA-Ds positive	38	39	0.026	-	
Ds517-1T_2	T-DNA-Ds negative	14	13	0.077	-	
Total		**52**		**0.103**	**3.841**	**yes**

Where x^2 cal. indicates the calculated Chi Square value
x^2 0.05,1 shows the value from Chi Squared table at p value 0.05 at 1 degree of freedom

7.3.1 Southern blot analysis of Ds517-1 T_2

A probe containing the complete ORF from NCED1 gene was prepared and hybridised to the blot containing restriction digested (*Spe-I and Nde-I*) DNA from several Ds517-1 T_2 plants positive for the *Ds* element. Southern blot analysis revealed two bands representing T-DNA-Ds elements in all T_2 progeny derived from Ds517-1 line (Figure 7-3).

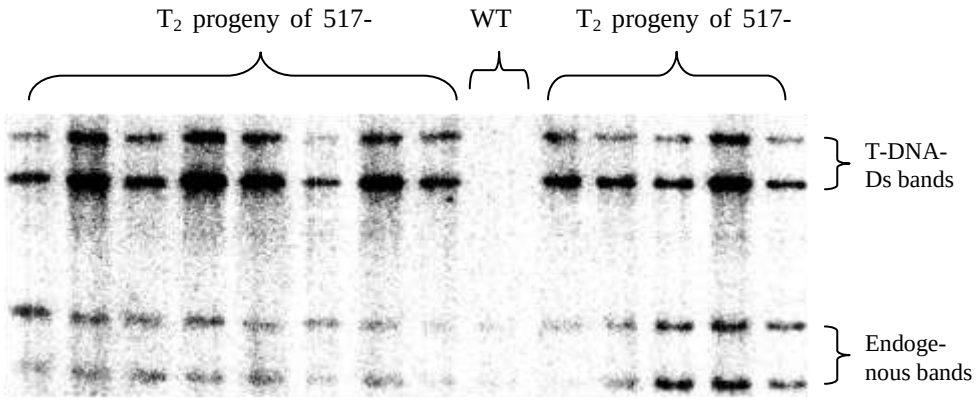

Figure 7-3. Southern blot analysis of T_2 progeny of parent Ds-lines (Ds517-1). The blot was hybridized with *LeNCED1* probe containing the complete ORF of NCED1 gene. The T_2 progeny of 517-1-lines containing the *Ds* element used in Southern blot had only two T-DNA-Ds bands. wild type was used as a control.

7.4 T-DNA-Ds element did not affect the leaf g_s in Ds517-1 T_2

It was important to establish if the two non-transposed T-DNA-Ds elements detected in the Ds517-1 T_2 line had a role in reduction of the g_s, and therefore to understand the extent to which the *Tr-Ds* rather than the original T-DNA-Ds element had influenced the g_s in the lines screened.

To investigate the effect of the two T-DNA-Ds elements present in the Ds517-1 T_2 (Figure 7-3) on the g_s, the plants were kept under controlled environment with wild type and sp5 as controls (Figure 7-4).

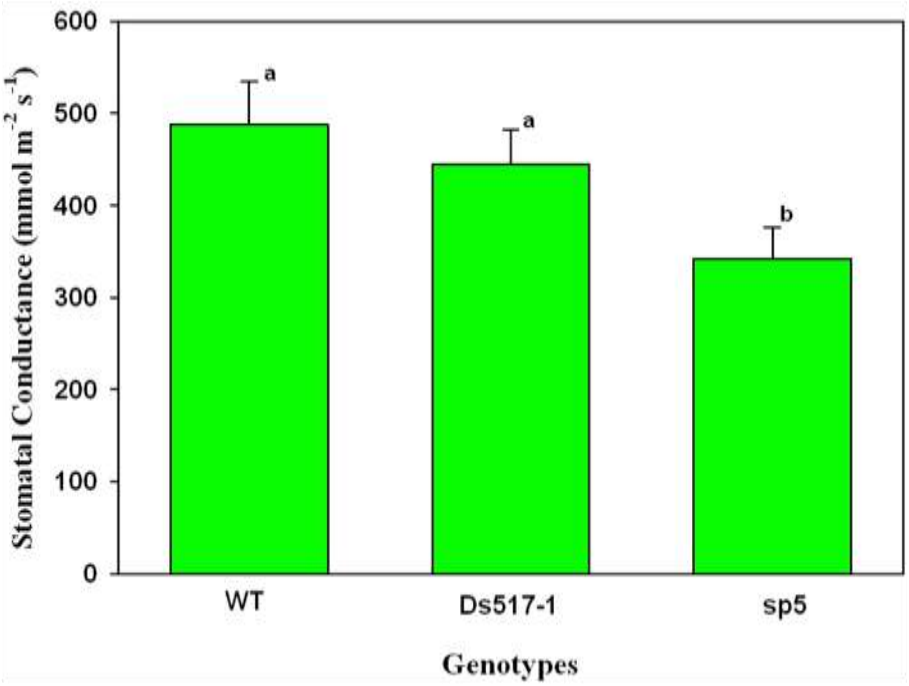

Figure 7-4. Leaf stomatal conductance of T_2 progeny of line Ds517-1 (selected through PCR for the T-DNA-Ds element) under controlled environmental conditions. Letters 'a' and 'b' represent the statistical significance among different lines; similar letters indicate no statistical difference in the g_s at $p=0.05$. Error bars represent the standard error of means. n=6.

The g_s data (Figure 7-4) revealed that there was a significant difference between wild type and sp5 plants ($p<0.001$) but no statistical difference could be found in the Ds517-1 T_2 and wild type plants. Although it had 7% lower g_s than the wild type plants, statistically there was no significant difference between the two genotypes. Further, *Ds*-lines had statistically higher g_s than sp5 plants.

Due to the lack of availability of the individual plant used for the cross (i.e. *sAc × Ds)*, there was a remote possibility that the individual parent line (Ds517-1) contained an additional T-DNA-Ds band that was not detected in the siblings. To avoid any confusion about T-DNA-Ds element copy number and their respective role in the g_s in each of the genotypes, Southern hybridization was carried out for T-DNA-Ds elements using a part of nptII gene as a probe that is present in the T-DNA-Ds but not the *Tr-Ds*. The genomic material used in this blot belonged to $102F_3$, Ds517-1-T_2 and wild type plants. Results in Figure 7-5 illustrated that only two copies of T-DNA-Ds element were present in both $102F_3$ and the Ds517-1-T_2 (progeny from selfing of the Ds517-1), none of the *Tr-Ds* bands identified on the Southern blot in line $102F_3$ (Chapter-5 Figure 5-5) was hybridized to the nptII probe, confirming that they were not T-DNA-Ds elements. As there were no T-DNA-Ds elements present in the wild type plants, no bands appeared on the blot. This data further reiterated that the reduced g_s was likely due to *Tr-Ds* elements rather than T-DNA-Ds elements. Also, this data is applicable to all F_3 families as they are all derived from the same Ds517-1 T-DNA integration event (described in Chapter 5 and 6).

Further, an attempt was made to remove the T-DNA-Ds element from *Tr-Ds* element in genotype $102F_2$(Ds+), this has been described below.

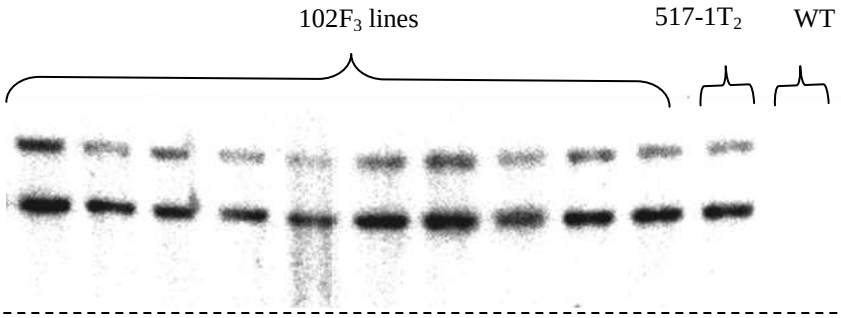

Figure 7-5. A Southern blot analysis of genotype $102F_3$ lines (self pollination of $102F_2$ genotype) revealed only two copies of npt-II:: Ds1::histoneH2A::*LeNCED1*::Ds2. There is no nptII gene present in the wild type plants hence no band appeared on the blot

7.5 Segregation of the *Tr-Ds* and T-DNA element

The *sAc/Ds* system allows the repositioning of the transgene and allows the segregation of the gene of interest within the transposon away from the T-DNA containing the antibiotic resistance gene (Cotsaftis et al., 2002). However, the segregation of these elements depends on the genetic distance between the original site of insertion and transposition of the *Ds* element carrying the gene of interest.

This section describes the segregation of the antibiotic resistance gene present in T-DNA-Ds away from the *Tr-Ds* element present in genotype $102F_2$(Ds+) through backcrossing with wild type (Ailsa Craig $Tm2^a$) plants.

The genotype $102F_2$(Ds+) was cross pollinated with wild type (Ailsa Craig $Tm2^a$). This BC_1F_1 progeny was genotyped by using primers *Ds1For2* and *notRev5* for the presence or absence of the *Ds* element (839 bp). To test the presence of T-DNA-Ds element, primer pair *Ds-T-DNA For1* and *Ds-T-DNA-Rev1* was used. The resultant amplicon was 399 bp, confirming the presence of T-DNA-Ds element.

In all, 81 BC_1F_1 plants were genotyped but none were found to have lost the T-DNA-Ds, element and all plants were found to contain the *Ds* element. This showed that 102-130F_3(Ds+) plant used in the cross with Ailsa Craig $Tm2^a$ must have been homozygous for the T-DNA-Ds and *Tr-Ds* elements.

7.5.1 Loss of T-DNA-Ds element in BC_1F_2 progeny (selfing of BC_1F_1)

In order to obtain segregation and recombination events, the heterozygous BC_1F_1 plants were either selfed or back-crossed again with Ailsa Craig $Tm2^a$. In total, 45 plants obtained from selfing of 35BC_1F_1 plant (35BC_1F_2; accession AT2489) were genotyped for the *Ds* element. Results showed that 38 plants retained the *Ds* element while seven plants lost it through segregation (Table 7-2). The chi-hsquare value calculated (2.15) indicated that the ratio was not significantly different from 3:1 (χ^2 = 3.84 at P = 0.05), which suggested the parent plant (35BC_1F_2; accession AT2489) was heterozygous for the *Ds* element.

The 38 plants containing the *Ds* element were further screened for T-DNA-Ds element. The results showed that two plants namely, 09-BC_1F_2 and 21-BC_1F_2 lost the T-DNA-Ds elements, but retained a *Tr-Ds* element (Figure 7-6). The chi-square test was carried out which showed that both T-DNA-Ds and *Tr-Ds* elements were genetically linked (Table 7-3) as they chi square value calculated (11.31) was significantly different from χ^2 (5.99) at P=0.05 (Table 7-3). The pedigree diagram of different lines obtained from genotype 102F_2 (Ds+) is shown in Appendix-III and detail about various genotypes used during the experiments is given in Table 7-4.

Table 7-2 Calculation of segregation ratio of *Ds* element in genotype $35BC_1F_2$ by using Chi square test

GT	Bands Present	Number of plants observed	Number of plants expected	Chi squared value observed	Chi square value expected	ratio
$35BC_1F_2$-(T-DNA-Ds+)	T-DNA-Ds + *Tr-Ds* + endogenous	38	33.75	2.15	3.84	3:1
$35BC_1F_2$-(T-DNA-Ds-)	Endogenous only	7	11.25			
Total		**45**	**45**			

7.5.2 Stomatal conductance measurements of *Tr-Ds* plants

In order to measure the effect of loss of T-DNA-Ds element on g_s in *Tr-Ds* plants, cuttings were taken from 09-BC_1F_2 and 21-BC_1F_2 plants to grow multiple uniform plants for the experiment. This also saved time without going through a prolonged process of seed bulking and re-growing the $35BC_1F_2$-T-DNA-Ds- plants.

Table 7-3 Calculation of segregation ratio of *Ds* element in genotype $35BC_1F_2$ by using Chi square test

GT	Bands Present	Number of plants observed	Number of plants expected	Chi squared value	Chi square at 0.05	ratio
$35BC_1F_2$-(T-DNA-Ds+)	T-DNA-Ds + *Tr-Ds* + endogenous	36	33.75	11.31	5.99	
$35BC_1F_2$-(T-DNA-Ds-)	*Tr-Ds* only	2	8.44			
Wild type	Endogenous only	7	2.81			
Total		**45**	**45**			

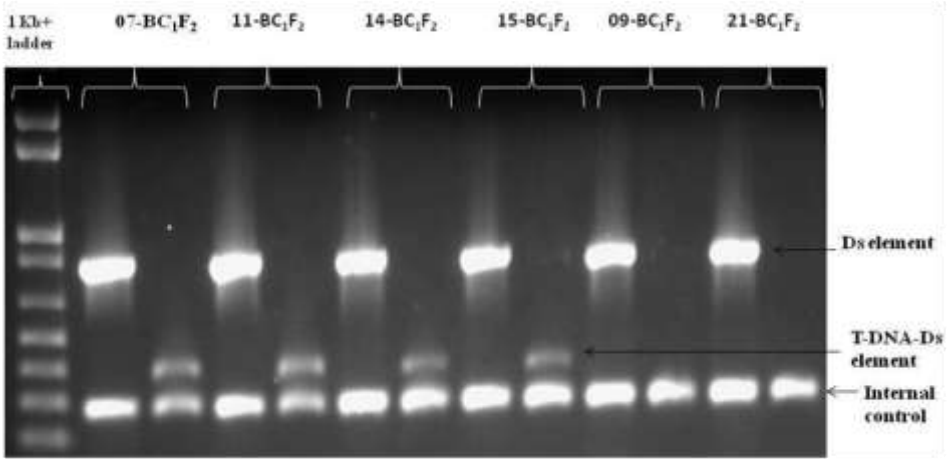

Figure 7-6. Gel image illustrating the segregation of T-DNA-Ds element and Tr-Ds element in genotype $102F_2$(Ds+). The *Ds* element (839 bp) was amplified by using primer pair *Ds1For2* and *notRev5*, these primers were unable to differentiate between T-DNA-Ds or Tr-Ds elements. The T-DNA-Ds element was amplified by using primers Ds-T-DNA-For1 and Ds-T-DNA-Rev1 (399 bp), while the 'internal control' (*phytoene synthase*) was amplified by using primers *phytoene synth-F* and *phytoene synth-R*. Note, this gel image contains pcr product from two different reactions.

Table 7-4 Description of genotypes used during g_s measurements in $35BC_1F_2$-T-DNA-Ds- plants

Genotype	Elements present
$102F_2$(Ds+)	T-DNA-Ds and *Tr-Ds*
$35BC_1F_2$-T-DNA-Ds+	Backcrossed progeny from $102F_2$(Ds+) contained T-DNA-Ds and *Tr-Ds* elements
$35BC_1F_2$-T-DNA-Ds-	Lost T-DNA-Ds element; only contained *Tr-Ds* element
Wild type	Nil

Approximately 6-week old plants were grouped in four blocks each containing two plants from the same genotype. The g_s data was recorded five times during the day in a Weiss room. The comparisons were made between plants containing the T-DNA-Ds element ($102F_2$(Ds+); its backcrossed progeny ($35BC_1F_2$-T-DNA-Ds+), ($35BC_1F_2$-T-

DNA-Ds-) and wild type (BC_1F_2 that lost the T-DNA-Ds element) plants (Genotype detail described in Table 7-3).

The g_s results (Figure 7-7) showed that $35BC_1F_2$-T-DNA-Ds- plants had the lowest g_s (171.5 mmol m^{-2} s^{-1}) significantly lower ($P<0.01$) from the wild type (390.9 mmol m^{-2} s^{-1}) and the $102F_5$(Ds+) plants (241.4 mmol m^{-2} s^{-1}). However, there was no difference in the leaf g_s of $35BC_1F_2$-T-DNA-Ds+ and $35BC_1F_2$-T-DNA-Ds- plants. These results suggested that the loss of T-DNA element had no effect on the plant g_s.

Moreover, these plants were used for seed bulking so that further experiments could be carried out in future in the absence of T-DNA element.

7.5.3 Reactivation of *Tr-Ds* element

The 35 BC_1F_2-T-DNA-Ds- plants (09-BC_1F_2 and 21-BC_1F_2) were cross pollinated again with *sAc* plant to reactivate the *Tr-Ds* element. The F_1 (*Tr-Ds* × *sAc*) seeds have been collected (accession AT2490) and screened for the presence of both *Tr-Ds* element and *sAc* element as previously described in chapter-3 section 3.2.1, to ensure successful transposition of the *Ds* element. The F_2 seeds from this cross were obtained by self pollination (accession AT2484) and potentially harbor the transposed *Tr-Ds* element without the presence of T-DNA element.

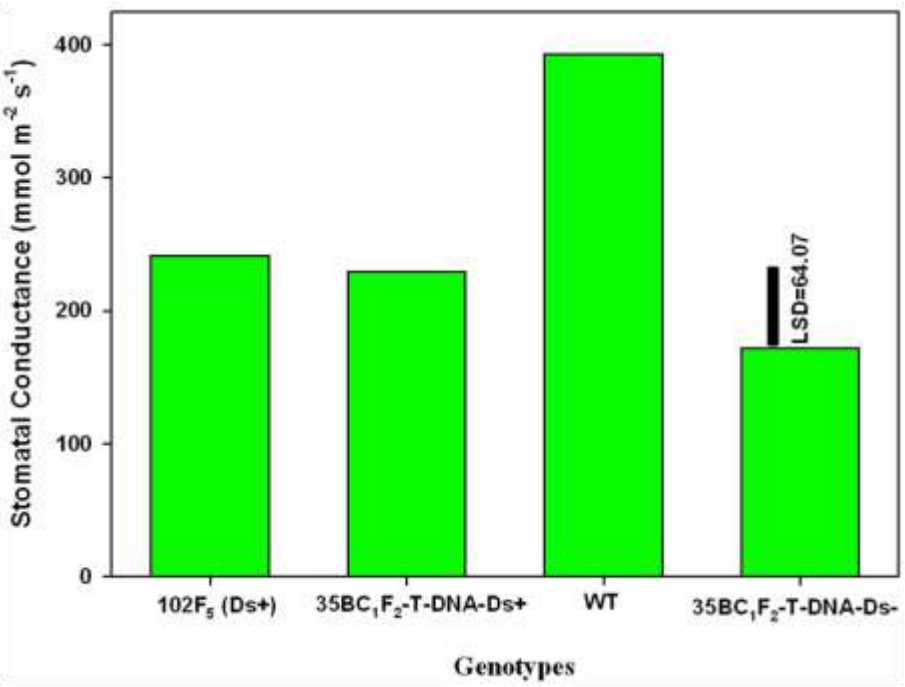

Figure 7-7. Leaf stomatal conductance of the genotype $102F_2$(Ds+) and its derivatives under controlled environmental conditions. Genotype $102F_5$(Ds+) represents the progeny derived from genotype $102F_2$(Ds+); Genotype $35BC_1F_2$ -T-DNA-Ds+ represents the genotype backcrossed with wild type (Ailsa Craig $Tm2^a$) that retained the *Ds* element; wild type plant shows the genotype where the *D*s element was lost through segregation and $35BC_1F_2$-T-DNA-Ds- shows the genotype that lost the T-DNA but retained the *Tr-Ds* element. The LSD value shows the least significant difference between the genotypes at $p<0.05$. Where n=6.

7.6 Discussion

The data showed that the transgene was present in a single complex locus because the presence or absence of the *Ds* element was segregating 3:1 (Table 7.1), but there were two separate bands evident on Southern Blots. In this study, the parent plant containing the T-DNA-Ds element (Ds517-1 T_2) had a complete set of apparatus for *LeNCED1* expression; Histone H2A is constitutively expressed in the plant genome and its extent of expression is low in tomato genome (Jones, 2007). The seeds from Ds517-1 T_2 did not germinate on water alone (Figure 7-2) and a very high rate of norflurazon

(6.50 μM) was required to germinate the seeds. It was also observed that the only seeds germinating on water alone were those that had lost the T-DNA-Ds element by segregation. This demonstrated that the T-DNA-Ds elements increased the seed dormancy in Ds517-1 T_2 probably through over accumulation of ABA in the embryo. Poor seed germination was previously observed in plants overexpressing *LeNCED1* gene (Thompson et al., 2000).

The leaf g_s data indicated no significant differences between the Ds517-1-T_2 and wild type plants, although the stomatal conductance was 7% lower in case of Ds517-1-T_2 (Figure 7-4). This reduction might be due to the presence of two copies of T-DNA-Ds (Figure 7-5) elements containing *LeNCED1* compared to the wild type plants, or it might be due to experimental variation alone or due to integration of *LeNCED1*transgene with tissue specific pattern of expression affecting seed germination but not the g_s.

The results from these experiments revealed that it is not always necessary for a genotype growing slower to have a reduced g_s and plants or vice versa. As the plant material used in this study from Ds517-1 T_2 was visually slow growing (no data on growth was recorded) with pale leaf colour compared to the wild type plants but they still had similar g_s to the wild type plants.

7.6.1 Production of antibiotic resistance gene free plant background

At present there is no evidence available regarding the horizontal transfer of antibiotic resistance genes used for plant transformation into pathogenic microorganism, mammals including humans (Ryffel, 2011) but the environmental activists claim the

possibility of horizontal transfer of these genes to microorganisms and humans through the food chain.

The two way transposon based (*Ac/Ds*) transformation of the plant material allows the repositioning of the transgene and allows the segregation of the gene of interest within the transposon away from the T-DNA containing the antibiotic resistance gene (Cotsaftis et al., 2002). The results shown in Figure 7-6 indicate that the two-way transposon based system (*sAc*×*Ds*) can be successfully employed to remove the antibiotic resistance gene, a key objection of GM skeptics towards acceptability of GM crops. However, this is important to consider that the removal of T-DNA-Ds element will depend on the genetic distance between the original T-DNA insertion and the *Tr-Ds* element. The greater the distance between the two elements more will be the chances of their segregation. But in case of transposons the *Ds* element containing the gene of interest usually transposes to a linked locus (Dooner and Belachew, 1991),further, the line used for cross pollination with the wild type plants must have been homozygous, this might be the reason that the BC_1F_1 progeny did not produce any plants that lost the T-DNA-Ds element.

It was also observed that the loss of T-DNA-Ds element did not affect the g_s as the plant that lost the T-DNA-Ds element ($35BC_1F_2$-T-DNA-Ds-) had statistically similar g_s to the plants retaining both T-DNA-Ds and *Tr-Ds* elements ($35BC_1F_2$-T-DNA-Ds-) As shown in figure 7-7. The reduced g_s in $35BC_1F_2$-T-DNA-Ds- plants might be due to the way the plants were propagated, as described earlier that these plants were grown from the cuttings, whereas. $35BC_1F_2$-T-DNA-Ds- plants were grown from the seed. The significantly higher g_s in $102F_2$(Ds+) plants compared to the $35BC_1F_2$-T-

DNA-Ds- might be due to a different genetic background of the two genotypes (Figure 7-7).

However, it will be interesting to know if the loss to T-DNA-Ds element influences the reactivation of the *Tr-Ds* element and if so up to what extent. It will also be useful to know about the effect on loss of T-DNA-Ds element on plant phenotype especially g_s, its ability to gain DW and most importantly WUE_p.

The findings during this study and studies previously carried out by (Thompson et al., 2000, Qin and Zeevaart, 2002, Hu et al., 2010) found that overexpression of NCED resulted in increase in seed dormancy and slow plant establishment (Thompson et al., 2000) . This was a potential set back that could deter growers. In order to overcome this, some new chemicals of NCED inhibitors were designed and used in tomato seeds with moderate to strong seed dormancy. This has been described in Chapter-8.

Chapter-8 Use of hydroxamic acids to improve seed germination and seedling establishment in high ABA genotypes

8.1 Introduction

A seed contains a fully mature embryo that enables it to survive adverse environmental conditions and allows the continuation of survival of a plant species (Koornneef et al., 2002). At the start of germination, a seed enters a phase of complex physiological changes that are triggered by imbibition with water under favourable internal and external conditions, expansion of embryo cells, chemical breakdown of endosperm through hydrolytic enzymes activated by the germinating embryo itself (Brown and Morris 1890), rupturing of the testa, and emergence of the radicle (Kucera et al., 2005).

However, sometimes seeds are unable to germinate even under favourable environmental conditions; such seeds are called dormant seeds. It is a temporary block to complete germination of a viable seed under conditions otherwise suitable to complete germination (Bewley, 1997, Finch-Savage and Leubner-Metzger, 2006, Finkelstein et al., 2008). Various dormancy mechanisms have evolved to allow seeds to germinate under the most appropriate environmental conditions and at the most appropriate time in the season to allow successful completion of the plant life-cycle.

8.1.1 Role of Abscisic acid in promoting seed dormancy

Seed germination has been suggested to be under the control of two independent processes, in the first phase reserves are mobilized and the second phase involves the elongation of the embryo axis (Pritchard et al., 2002). However, several factors can be responsible for blocking the completion of seed germination. It can be in the form of a

hormone such as ABA or a physical barrier such as endosperm or seed testa. In case of tomato seeds, the endosperm is a constraining structure for the embryo growth and radicle extension (Bewley, 1997) and the resistance caused by these enclosing structures is reduced just before the radicle emergence (Watkins and Cantliffe, 1983). This is achieved by cell wall hydrolysis for example hemicellulases produced within, and secreted by, the endosperm (Bewley, 1997).

There is considerable evidence available that ABA regulates testa development during embryogenesis, which can play important role in maintaining seed dormancy the evidence came from the ABA deficient mutant *sitiens* whose testa is only one cell thick compared to the wild type with five cell layers (Hilhorst and Downie, 1996). Some of the high ABA genotypes used during this study also had problem with post-germination cotyledon emergence from seed coat (described in Chapter-6). This gives strength to the argument that ABA can cause a physical barrier in seed germination and early plant establishment.

Dormancy may also depend on the balance between ABA and gibberellic acid (GA) concentrations in the seed, which in turn depends on their rates of biosynthesis and catabolism. For example, increased rates of ABA catabolism would promote germination by increasing the concentration ratio of [ABA]:[GA] (Ali-Rachedi et al., 2004, Cadman et al., 2006). If the embryo in question is more sensitive to the presence of ABA, then the dormancy after maturation is increased (Walkersimmons, 1987).

Mainly ABA is catabolised by an oxidation reaction at C-8′ resulting in 8′-hydroxy-ABA which is converted to phaseic acid (PA). This oxidation is carried out with the help of ABA-8′-hydroxylase encoded by CYP707A (Kushiro et al., 2004). Further, ABA responsive elements and their binding transcription factors regulate the

ABA responses in seeds which can determine the seed dormancy (Cadman et al., 2006). The transcriptionally regulated genes include ABI3 (ABA insensitive -3)(Koornneef et al., 1984) and DOG1 (delay of germination-1) (Alonso-Blanco et al., 2003).

8.1.2 Use of chemical approach to inhibit ABA biosynthesis in plants;

As previously described in Chapter-1, ABA is formed by the oxidative cleavage of epoxycarotenoids (Schwartz et al., 1997). The transgenic plants produced here by Dr. Andrew Thompson's Lab. at University of Warwick, over expressing *LeNCED1* showed increased water use efficiency but with some deleterious effects too e.g., increased seed dormancy (Thompson et al., 2000, Tung et al., 2008) and delayed seedling establishment. Overexpression of *LeNCED1* increased seed ABA contents in sp5 and sp12 (previously named D9) by approximately three-fold (Thompson et al., 2000), these results also demonstrated that sp5 seed had increased dormancy than sp12 and fail to germinate on water alone, in order to improve their seed germination norflurazon (NZ) was used.

Fluridone and norflurazon (NZ) are pre-emergence herbicides that inhibit phytoene destaurase and so block the synthesis of all carotenoids (Sandmann et al., 1997), including the epoxycarotenoid substrates of ABA biosynthesis (Popova and Riddle, 1996). Fluridone and NZ can therefore stimulate seed germination by reducing dormancy due to inhibition of ABA biosynthesis (Grappin et al., 2000, Ali-Rachedi et al., 2004). However, the imbibition of seeds in the presence of NZ or fluridone can cause bleaching in developing cotyledons by depletion of the photoprotective carotenoids and the subsequent degradation of the photosynthetic apparatus (Breitenbach et al., 2001), and seedling death.

The NCED inhibitors previously used include abamine and its second generation derivative abamine-SG. The abamine inhibitor resulted in a slight increase in radicle elongation in cress seedlings (Han et al., 2004) and to the authors knowledge there are no reports of an effect of abamine or abamine-SG on the seed germination or seedling establishment. This necessitated the development of more specific inhibitors of ABA biosynthesis, for example targeting NCED, a key rate limiting enzyme in ABA biosynthesis (Thompson et al., 2007), which could be used to stimulate germination in high ABA plants.

The carotenoid cleavage dioxygenases are the enzymes which catalyse the oxidative cleavage of carotenoids at various chain positions to form a variety of apocarotenoids, and NCED is a sub-class of CCDs. The design of these inhibitors was based on a structural mimic of the substrate of the CCDs, incorporating an Fe-chelating hydroxamic acid group within its active site (Figure 8-1). In each chemical, the positioning of the iron chelating hydroxamic group was changed by the use of an aromatic ring, so that it matched the distance within the carotenoid substrate between the proximal cyclic end group and the cleavage site in the particular carotenoid (Sergeant et al., 2009). These inhibitors were designed in collaboration with Professor Tim Bugg from Chemistry department, University of Warwick and the main objectives of designing these inhibitors was to find out the functions of diverse members of the CCD gene family.

These chemicals were tested *in vitro* for inhibition of enzymes *LeNCED1* and *LeCCD1a* which cleave at the 11′-12′ and 9,10 position of carotenoids respectively . The results showed that the hydroxamic acids D4, D7 and D8 had 1.5-2.0 folds more inhibitory activity against NCED than abamine (Sergeant et al., 2009). This prompted

the use of these chemicals in this thesis to test their ability to break the seed dormancy and promote germination and early plant establishment through inhibition of *LeNCED1*, a key regulatory enzyme in ABA biosynthesis.

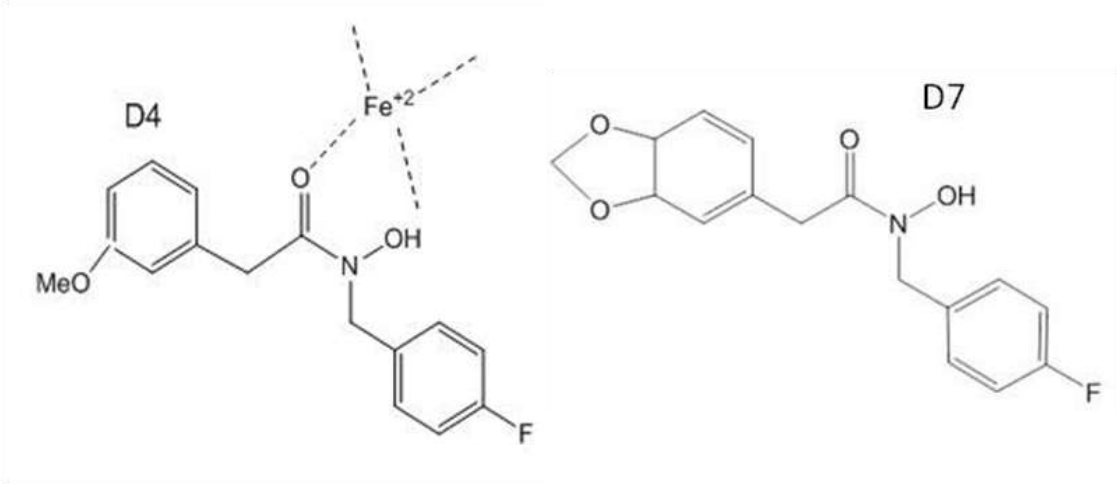

Figure 8.1. NCED inhibitors. The chemical structure of hydroxamic acids D4 and D7 showing chelation of the hydroxamic acid group to the Fe^{2+} present at the catalytic centre of NCED.

8.2 Materials and Methods

8.2.1 Preparation of hydroxamic acid solutions

Hydroxamic acids could only be dissolved in organic solvents hence they were dissolved in 100% DMSO. A stock solution was prepared for each hydroxamic acid at 0.4 M and then stored in the dark at -20^{o}C. Stock solutions were diluted in water at the required concentration. The final concentration of DMSO in each treatment was adjusted to a constant 0.5% v/v.

8.2.2 Plant Material

8.2.2.1 Seed germination

Seeds were surface sterilised in 50 ml screw-cap centrifuge tubes (Falcon tubes) using 10% (v/v) household bleach (Domestos, Unilever, UK). Tubes were left on a rocking platform to shake gently for 15 minutes followed by rinsing with sterile dH_2O at least three times to remove the bleach. In Petri dishes, 15-20 seeds from each genotype were incubated on sterile filter papers (Whatman filter paper Grade-1, 8.5 cm diameter) soaked with 1.5 ml of aqueous solution containing the appropriate chemical concentration. These Petri dishes were kept in a plastic box containing moist tissue paper to maintain high humidity in order to avoid drying of the filter paper. The box was incubated at 25^{o}C in the dark. Germination was recorded on a daily basis and seeds were considered to have germinated upon the visible emergence of the radicle (Bewley, 1997).

Non destructive leaf area was measured by taking still images from a fixed position approximately 50 cm above the tray containing plants. These images were analysed using Image-J software. The mean germination time (MGT) was calculated by using a macro in Microsoft Excel, written by Hugh Rowse of HRI, Wellesbourne.

8.3 Results

8.3.1 Screening of hydroxamic acids on the basis of their ability to promote seed germination

As described previously in section 8.1.1, the seeds obtained from genotype sp5 and sp12 had nearly three times higher ABA contents than the wild type, further the seed from genotype sp5 had stronger dormancy compared to sp12 though no difference

in seed ABA contents could be detected in mature imbibed seeds of these two genotypes (Thompson et al., 2000). Normally sp12 seeds germinate on water without the need of NZ, but the germination on average is 8-10 days delayed compared to the wild type seeds, depending on the age of the seeds as the fresh seeds are more dormant than older seed batches (personal observation). In order to confirm that the seeds were viable and could be used in the germination experiments, NZ was used to at 3.3 µM (1 mg l^{-1}) and 6.58 µM (2 mg l^{-1}) in case of sp5. At this concentration of NZ no major photobleaching of cotyledons was observed in sp5 and sp12 seedlings.

In this experiment, 20 seeds were incubated with the appropriate chemical, each treatment was replicated twice. Hydroxamic acids D1-D7 (Sergeant et al., 2009) were tested for their effect on germination of sp12 seeds. This genotype was selected due to its moderate seed dormancy (i.e. higher than wild type but lower than sp5) (Thompson et al., 2007). The sp12 seeds incubated on 3.3 µM NZ started germinating 3 days after imbibition (DAI) and completed their germination 8 DAI (Figure 8-2a).

Seeds incubated on water + 0.5% DMSO (negative control treatment) did not start germinating until 8 DAI. All hydroxamic acids tested increased the final percentage of seed germination 17 DAI compared to the seeds incubated on water + 0.5% DMSO (Figure 8-2-A & B).

The results in Table 8-1 show that the minimum time to reach 50% seed germination (T_{50}) by using a hydroxamic acid was in case of D4 at 1 mM and maximum time to reach T_{50} was taken by D5 (16 days). The results in Figure 8-2 A &B and Table 8-1 showed that maximum seed germination percentage (95%) was obtained in D4 at 1 mM followed by D7, D2, D3 and D6, with 92.5, 82.5, 67.5 and 67.5, germination respectively, 17 DAI. With D4 and D7 more than 3 times higher percentage seed

germination was achieved compared to the water only treatment, on the other hand there was not much difference between D1 and water only treatment 17 DAI, and other compounds were intermediate. The results in Table 7-1 showed that the minimum mean germination time (MGT) taken by a hydroxamic acid was in D4 (12.0) closely followed by D7 at 12.3 days. It was clear that the final percentage germination showed the largest effect, so D4 and D7 appeared to mainly affect the number of seeds that were able to germinate, rather than the timing of germination. In contrast, NZ dramatically increased both MGT and final percentage germination.

Table 8-1: Seed germination of sp12 genotype by using different chemicals.

Genotype	**Sp12**		
Chemical	**T_{50}(days)**	**Max. Germination (%)**	**MGT (days)**
Water only	-	27.5	12.5
Nz at 3.3 µM	7	100	6.2
D1 at 1mM	-	35	14.3
D2 at 1mM	15	82.5	13.7
D3 at 1mM	15	67.5	13.5
D4 at 1mM	14	95	12.0
D5 at 1mM	16	50	13.4
D6 at 1mM	15	67.5	12.7
D7 at 1mM	15	92.5	12.3

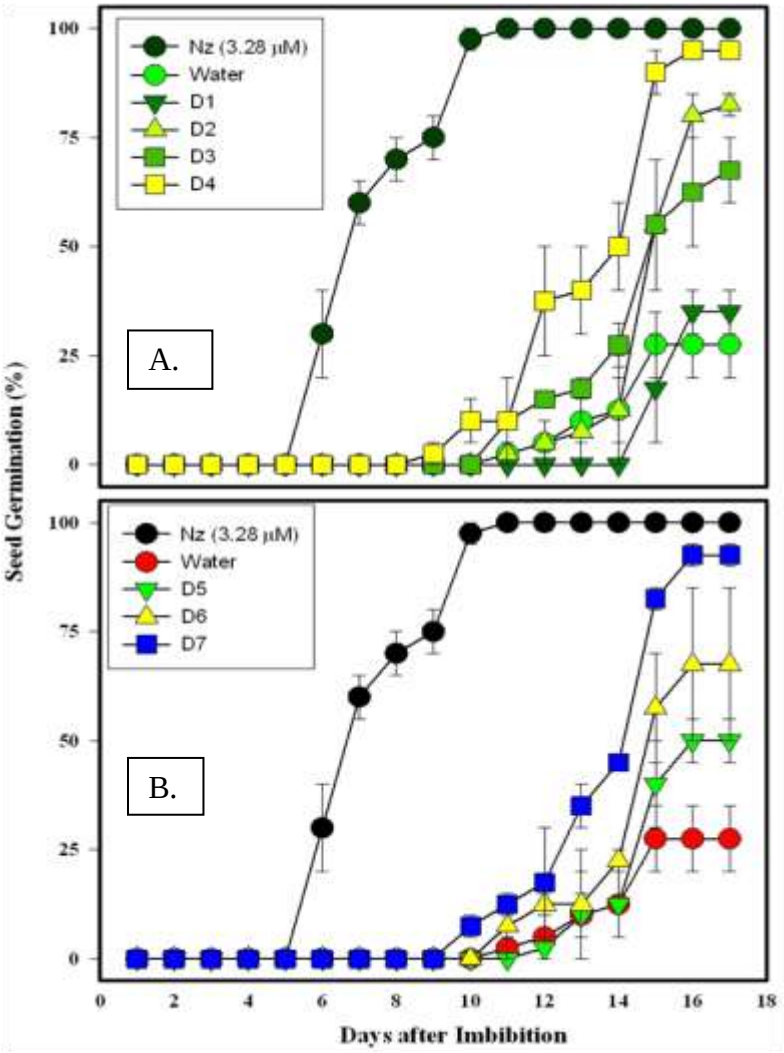

Figure 8.2. Seed germination of NCED overexpression line sp12 in the presence of various hydroxamic acids over a period of 17 days. Data was split into two separate figures (A&B) for convenience to interpret the results. Error bars indicate the range from two Petri dishes each containing 20 seeds. Nz, norflurazon; D1 to D7, names of hydroxamic acids (Sergeant et al., 2009). Each treatment contained 0.5% DMSO.

It was observed that the use of D7 caused cotyledon browning in the seedlings but D4 did not cause any damage to the seedlings hence D4 was selected for optimisation of concentration experiments for seed germination in wild type, sp5 and sp12 genotypes.

8.3.2 Response of germination to varying D4 concentration

As described in section 8.3.1, 1.0 mM D4 improved the rate of seed germination in sp12 (Figure 8-2 & Table 8-1). Next, the effect of varying concentrations of D4 on germination was investigated in wild type (cv. Ailsa Craig Tm2a), sp5 and sp12 seeds. The wild type were included in the experiment to evaluate if D4 helped in uniform seed germination of this genotype.

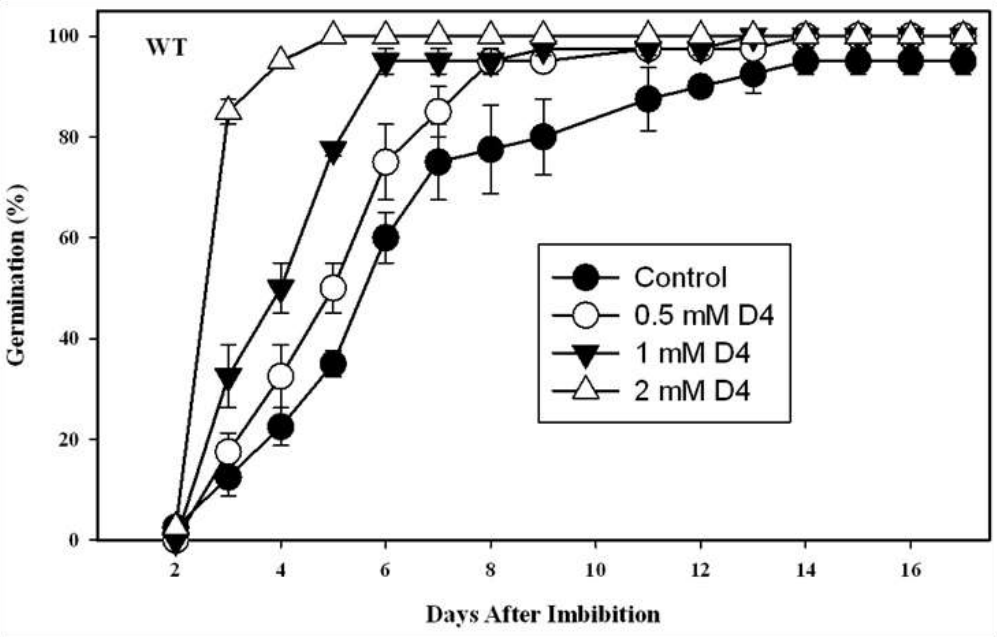

Figure 8.3. Effect of different concentrations of D4 on germination of wild type (cv. Ailsa Craig Tm2[a]) seeds. Error bars indicate range between two Petri dishes, each containing 20 seeds. Each treatment contained 0.5% DMSO.

In these experiments, the treatments included no D4 (water), 0.5 mM D4, 1.0 mM D4 and 2.0 mM D4. All the treatments contained 0.5% v/v DMSO. Results in Figure 8-3 showed that 100% wild type seed germination was achieved at 5 DAI when 2.0 mM D4 was used, whereas only 95% germination was achieved when no D4 was used even after 12 DAI. The results in Figure 8-4 and 8-5 revealed a strong negative correlation (r^2= -0.63; *p=0.003*) between the MGT and the concentration of D4 in the

wild type showing that with increasing concentration of D4 the MGT decreased. The results in Table 8-2 showed that with increase in D4 concentration T_{50} decreased from 6 days to 3 days with water alone and 2.0 mM D4, respectively.

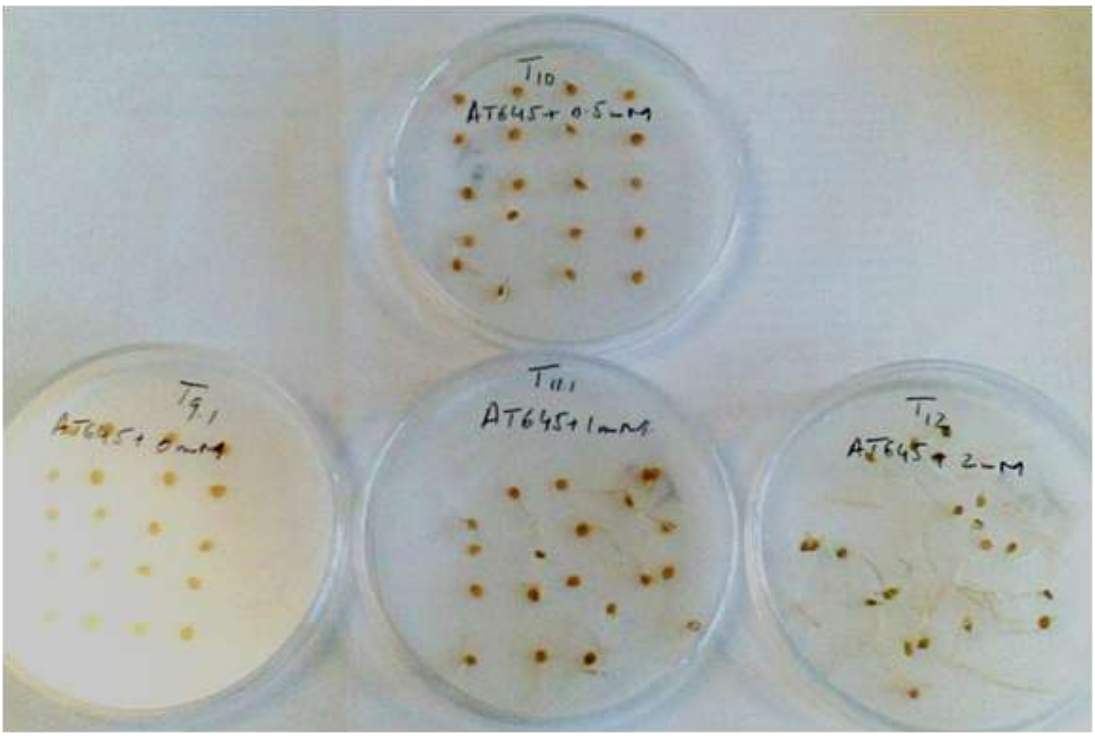

Figure 8.4. Seed germination of sp12 (accession AT645) under the influence of different concentrations of D4 as indicated on the Petri dish lids. 20 seeds were placed on moist filter papers and this picture was taken 12 days after imbibition.

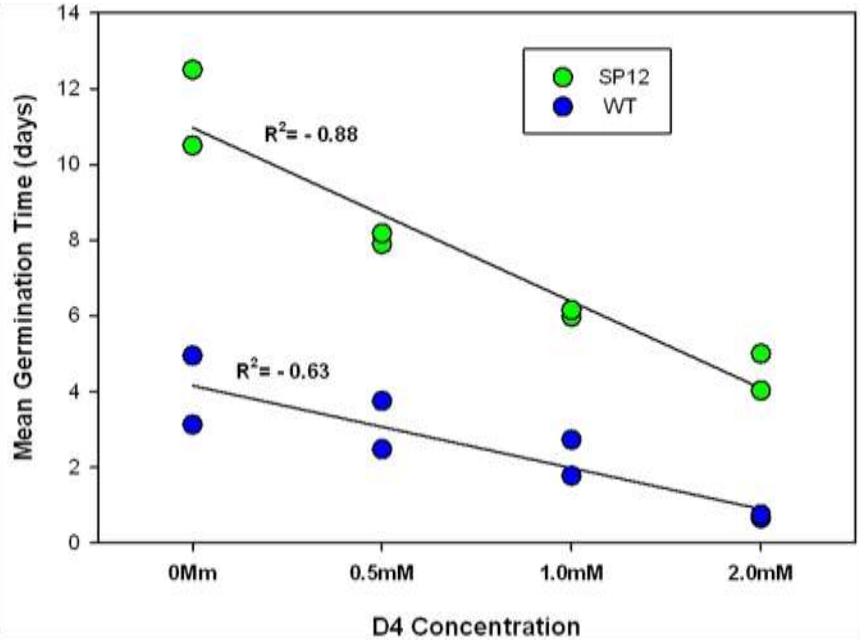

Figure 8.5. Relationship between the D4 concentration and mean germination time in wild type (Ailsa Craig Tm2a) and sp12. Where 'R^2' shows the coefficient of correlation. There were 20 seeds in each Petri dish and each treatment was replicated twice.

A further experiment was performed using sp12 seeds and a range of D4 concentrations (Figure 8-6). In this case sp12 seeds germinated very poorly on water alone, with only 2.5% germination achieved 13 DAI, and no further germination occurring at 18 DAI, while using D4 at 2.0 mM increased the final germination to 100%. However, there was a progressive response of germination to the increasing concentration of D4 with a strong negative correlation (r^2 = -0.88; *p<0.001*) observed between MGT and increasing concentration of D4 (Figure 8-4). With 2.0 mM D4, T_{50} was achieved 6 DAI, whereas, T_{50} with 0.5 mM D4 was recorded 10 DAI (Table 8-2).

Table 8-2: Seed germination of wild type and sp12 genotypes under the influence of different D4 concentrations

Genotypes	Concentration (D4)	T_{50} (days)	$Germination_{max}$ (%)	MGT
Wild type	0mM	6	95	4.03
	0.5mM	5	100	3.11
	1.0mM	4	100	2.25
	2.0mM	3	100	0.70
sp12	0mM	-	2.5	11.25
	0.5mM	10	90	8.04
	1.0mM	8	100	6.06
	2.0mM	6	97	4.51
sp5	0mM	-	0	-
	0.5mM	-	0	-
	1.0mM	-	5	-
	2.0mM	-	27.5	10.64
	NZ at 6.58 µM	1	100	1.50

For sp5 line, 100% seed germination was achieved with the application of NZ (6.6 µM) 2.0 DAI but no germination was achieved in control (water and DMSO only) (Figure 8-7). At 0.5 mM D4 had no effect on germination, but 5% seed germination was recorded with 1.0 mM D4 at 14 DAI. Further, at 2.0 mM D4, only 27.5% sp5 seeds germinated at 16 DAI. Thus, again there was a progressive increase in germination of sp5 seeds as the concentration of D4 increased.

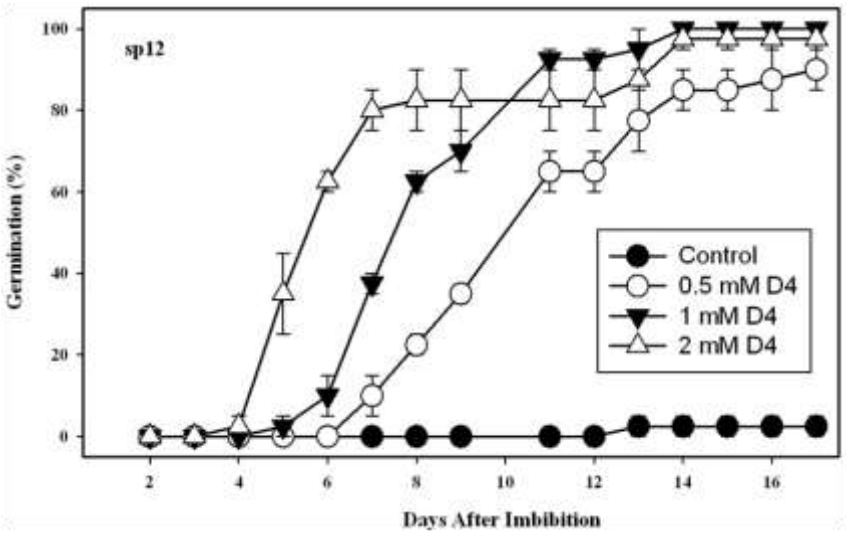

Figure 8-6. Effect of different concentrations of D4 on the seed germination of genotype sp12. Stock solutions were prepared in DMSO, each treatment contained 0.5% v/v final concentration of DMSO. Error bars indicate range of the mean from two Petri dishes, each containing 20 seeds.

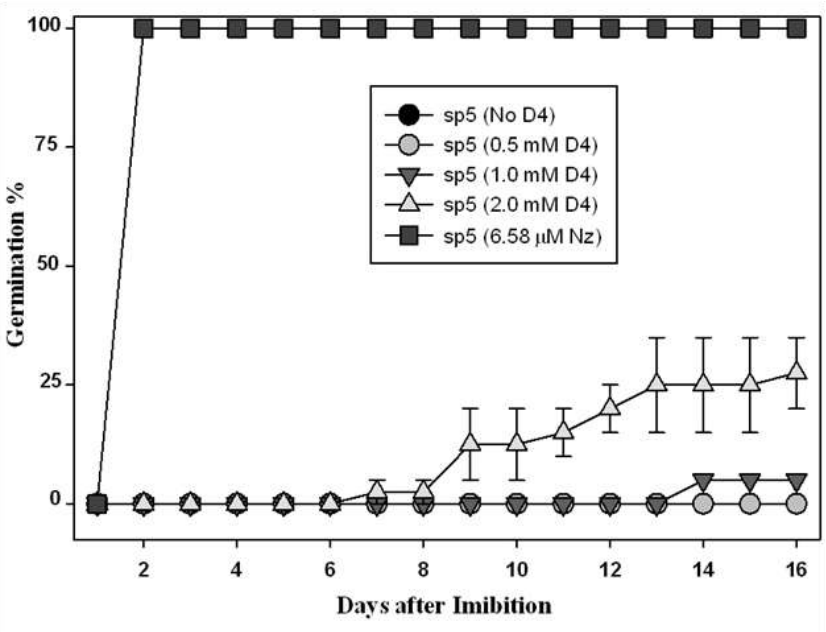

Figure 8.7. Effect of different concentrations of D4 on the seed germination of genotype sp5. Stock solutions were prepared in DMSO (Leake et al.), each treatment contained 0.5% v/v final concentration of DMSO. Error bars indicate range of means from two Petri dishes, each containing 20 seeds.

8.3.3 Effect of D4 application on seedling growth

As described above, the application of D4 improved the rate of seed germination of high ABA seed lines, it was an excellent candidate to increase agronomic utility of high ABA varieties. In this section, the aim was to evaluate the effect of D4 on seedling establishment post-germination. Seeds of sp12 were imbibed on water for seven days and then transferred to 2.0 mM D4 to promote synchronous germination. All seeds germinating on the same day were then transferred to compost at the stage when only radicles had emerged.

On seedling emergence from the soil, cotyledons were painted with a brush with either water or 2.0mM D4 once every morning for up to 10 days after seedling

emergence. The trays were then left under partially shaded conditions to avoid rapid evaporation of the solution.

A. B.

C. D.

Figure 8.8. Rate of plant establishment in sp12 genotype when painted with or without D4. Each tray contained 24 plants and each plant was painted with D4 or water every morning till harvest. A&B, 2 days from emergence painted with water or 2.0 mM D4, respectively. C&D, 8 days after emergence painted with water or 2.0 mM D4, respectively. Each treatment contained 0.5% v/v DMSO.

8.3.3.1 Application of D4 slightly improved the rate of LA expansion

At different stages of sp12 plant development, there were no statistical differences in LA between the two treatments (i.e. painting with water alone or painting with D4) (Figure 8-9a). The lowest probability was observed at six days after seedling emergence (*P = 0.051*); this *P* value is suggestive of an effect. Similarly, the results

obtained from destructive harvest of plants revealed that statistically there was no difference in plant dry biomass, although there was more dry biomass from the leaves painted with D4 by 16.5% (*P = 0.08*) (Fig 8-9).

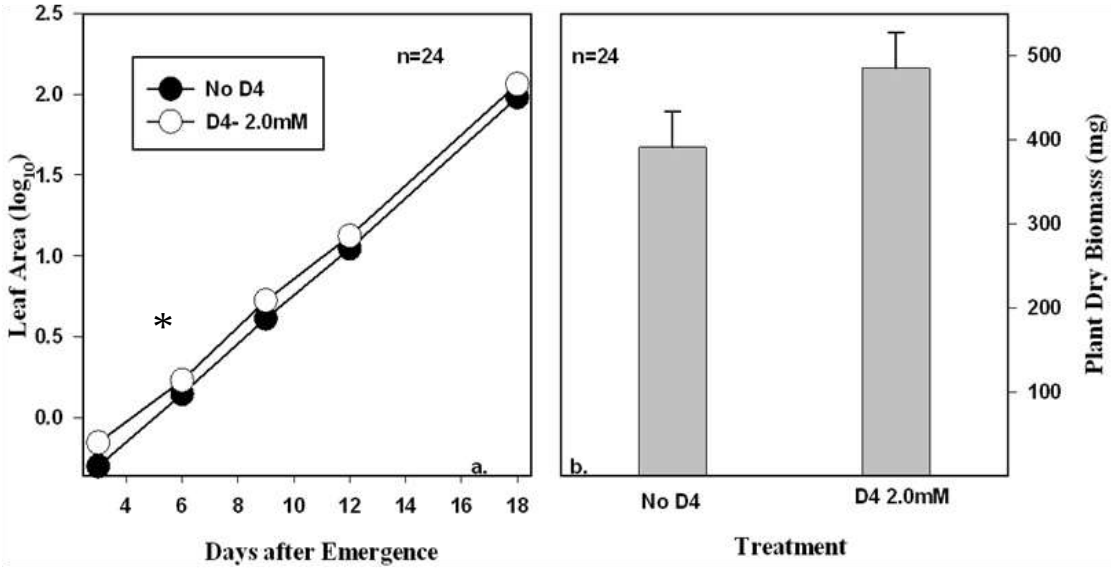

Figure 8.9. Effect of D4 on plant growth and biomass accumulation in sp12 seedlings. a. Log_{10} of total plant leaf area collected through non destructive and destructive measurements. b. Plant dry biomass gained over the period of 18 days of growth. Stock solutions were prepared in DMSO such that each treatment contained 0.5% final concentration of DMSO. Error bars indicates the range from two replications, where n = 2. The asterisk mark indicates suggestive difference in plant leaf area at *P=0.051*.

8.4 Discussion

The ABA biosynthesis inhibitor approach can be more useful compared to ABA mutants as it is dose dependent, the effects can be removed at any time and can be used in all types of plants. In order to develop more potent ABA biosynthesis inhibitors, hydroxamic acid were previously designed (Table 8-3) and their effects were evaluated on seed germination in constitutively high ABA genotype sp5 and sp12. Their role in early plant establishment was evaluated in sp12. The tomato seeds used during the

present study (sp5 and sp12) had strong seed dormancy (Thompson et al., 2000). For instance, sp5 seeds will not germinate under favourable germination conditions even after several weeks due to the overexpression of *LeNCED1* and accumulation of ABA (Thompson et al., 2000).

The *in vitro* inhibition of recombinant *LeNCED1* at 100 μM by the 4-hydroxyaryl hydroxamic acids D1 and D2 was 27 and 29% respectively, and this was similar to the *in vitro* activity of D4 and D7 (33% each) (Sergeant et al., 2009). However, the inhibitory action of these compounds against NCED, as determined by the seed germination test was only detectable in compounds D4 and D7, and not in D1 and D2. It is possible that the D4 and D7 compounds are more actively taken-up and or mobilized within the cell or their rate of inactivation or degradation was lower.

Table 8-3. Inhibition of recombinant *LeNCED1* enzymes using *in vitro* assays (adapted from (Sergeant et al., 2009))

Inhibitor				***LeNCED1* (11',12')**
Class	**Name**	**X**	**Y**	**Inhibition at 100mM (%)**
Aryl-C_2N	D1	4-OH	H	27
Aryl-C_2N	D2	4-OH	F	29
Aryl-C_2N	D3	3,4-$(OH)_2$	F	4
Aryl-C_2N	D4	4-Ome	F	33
Aryl-C_2N	D5	3,4-$(Ome)_2$	H	8
Aryl-C_2N	D6	3,4-$(Ome)_2$	F	18
Aryl-C_2N	D7	3,4-OCH_2O	F	33

The sp12 seeds are able to germinate on water alone, but may take up to several days to weeks before 100% seed germination is achieved. This wide range of times

required to germinate by the same genotype for different seed batches may be explained by the decrease in sensitivity of the embryo to ABA as the seed gets older (Farnsworth, 2004). The hydroxamic acid D4 has proved to increase the seed germination percentage and decreased the MGT and it can act as an effective inhibitor of ABA biosynthesis in sp12 seeds (Table 8-2; Figure 8-6). It was also encouraging to observe that with increasing concentration of D4 there was a decrease in MGT (Table 8-2). Higher concentrations of D4 were tried but the solubility of this compound was compromised above 2mM concentration.

On the other hand, little effect of D4 was observed on sp5 seeds. One of the possible reasons for this could be a higher seed ABA content in this genotype at certain times, although this was not apparent at 48 hours after imbibitions. When both sp12 and sp5 had three times more ABA than wild type (Thompson et al., 2007). It might be due maternal effects such as testa thickness as the ABA is known to increase testa thickness (Hilhorst and Downie, 1996), which might not allow D4 to penetrate to reach the embryo where bulk of metabolic activity occurs before germination or the time of *LeNCED1* expression 48 hours of imbibition might be different in sp5 and sp12 seeds.

It has been reported that more than 10% of total up-regulated genes in an embryo axis are involved in cell wall biosynthesis (Gimeno-Gilles et al., 2009) and ABA prevents the cell wall loosening (Bewley, 1997). The *LeNCED1* gene might have been expressed earlier in genotype sp5 than sp12, blocking the expression of the cell wall loosening and seed reserves mobilization genes resulting in stronger inhibition of germination (Figure 8-7).

In the current study it was noted that D7 increased the germination percentage and slightly reduced MGT at 1 mM in sp12 genotype (Figure 8-2, Table 8-1) but soon

after germination the radicles turned brown, though it did not cause the death of the seedlings in Petri dishes. Thus no further work was carried out with D7.

8.4.1 Importance of rapid plant establishment

After several glasshouse experiments, Thompson et al (2007) noted that sp5 and sp12 plants established 4-10 days slower than the wild type plants. However, the growth rate in these genotypes was similar or higher than wild type approximately 4 weeks after germination under well watered conditions (Thompson et al., 2007). However, initial rapid establishment can be a very useful trait especially under limited watered conditions by reducing the unproductive loss of water (Siddique et al., 2001) through direct exposure of moist soil to wind and sunshine. The slow plant establishment in these genotypes was due high endogenous level of ABA (Thompson et al., 2007), which we hypothesized could be modified with the application of hydroxamic acids. It was decided to paint the D4 dissolved in 0.5% v/v DMSO for rapid plant translocation on to the newly emerging leaves (Burns et al., 1969) in an attempt to minimize the growth reduction caused by ABA.

The results of painting of D4 on the cotyledons and leaves indicates of an effect on the rate of plant establishment measured as leaf area (Figure 8-8) or dry biomass accumulation Figure 8-9 a&b, respectively, though the differences were not quite significant at the 5% level. This suggested that the use of the novel *LeNCED1* inhibitor has potential agronomic value to improve crop growth and ground cover saving valuable irrigation water. This might be true for transgenic high ABA varieties if they were commercialised, or for conventional varieties that might have their canopy expansion limited in some circumstances by over-sensitive ABA signaling mechanisms. Such

chemicals could be used to improve germination for direct drilled crops grown under sub-optimal environmental conditions (e.g. spring maize germinated at lower temperature and lettuce grown under higher temperature). Hence the development of novel ABA inhibitors could be useful in the seed industry, and also for research purposes to study the role of ABA without the need for genetic intervention.

Therefore, it is concluded from this study that the hydroxamic acids inhibitors are promising lead compounds that could be used to counteract the effects of ABA in agronomic situations where the effects of ABA are undesirable. However, detailed study needs to be carried out involving more number of plants and genotypes, and different stress conditions in order to find the extent to which these compounds can be useful in inhibition of *LeNCED1* biosynthesis to help in uniform seed germination and rapid plant establishment.

Chapter-9

General Discussion

The research project described in this thesis used two main approaches to address the overall aim of developing means to produce ABA-mediated high WUE_p phenotypes with acceptable rates of germination and establishment. The first approach was to generate novel variation in patterns of ABA over-accumulation in tomato by transposition of the *Ds*(*Ds*1::H2A::*LeNCED1*::*Ds*2) element containing the *LeNCED1* gene with the help of *sAc* (stabilized activator element) as described in Chapter 3, followed by selection of tomato lines with a reduced stomatal conductance (g_s) and improved water use efficiency (WUE_p). The second approach was to test chemical inhibitors of the NCED enzyme to improve germination of NCED overexpression lines with known high WUE_p but with relatively mild to strongly dormant seeds.

9.1 The *sAc* induced transposition of the *Ds* element

Tomato plants containing *sAc* and *Ds* element were cross pollinated. This resulted in activation of the *Ds* element which transposed to different positions in the genome of F_1 (s*Ac* × *Ds*) plants. Southern blot analysis of F_2 lines showed unique transposition events (Chapter-4 section 4.2.7). This demonstrates that the two-way transposons based approach was successful resulting in the transposition and stable integration of *Tr-Ds* elements. Previous studies have shown that the *Ac/Ds* system can be used in different organisms with varying success rates in terms of *Ds* activation by s*Ac* element and its stable integration in the genome depending on its site of integration (Koprek et al., 2001, Cotsaftis et al., 2002) and the activity of these elements.

This thesis also shows that the two component transposon-based system (*sAc*×*Ds*) can be a useful tool to produce plants with variation in ABA content, without the difficulties associated with selecting high ABA plants during tissue culture, such as intensive labour, induction of transgene promoters by high sucrose concentration in the growth media resulting in excessive ABA biosynthesis and poor growth in artificially high humidity (Thompson et al., 2000).

The transposition of *Ds element*s is known to occur quite randomly, although it is preferential (Moreno et al., 1992) to insert near translation start codons in genes (Pan et al., 2005) and also to transpose to sites genetically linked to the site of excision, (e.g. linked to the T-DNA as observed during the present study). The PCR results of *Tr-Ds* elements showed that the genotypes $59F_2$ and $102F_2$ had *Tr-Ds* elements linked to T-DNA elements, with a single complex locus segregating at 3:1 (chapter-5, section 5.3.3.2 & section 5.3.2.3) whereas in genotype $116F_2$ the segregation ratio of *Tr-Ds* elements was 9:3:3:1 indicating that there were two unlinked loci containing *Tr-Ds* elements (Chapter-5 section 5.3.1.2). Similar observations were made by Bancroft and Dean (1993), who reported that up to 68% of the inherited *Ds element*s transposed to the genetic loci which were linked to the original site of insertion in *A.thaliana* (Bancroft and Dean, 1993).

The effects of these unique transposition events in genotype $59F_4$(*Ds*+) and $102F_5$(*Ds*+) were determined by growing these genotypes simultaneously with the wild type and sp5 control plants. The F_3 progeny of genotype $59F_2$ and $102F_2$ exhibited symptoms of putatively high ABA such as reduced g_s (usually a good indicator of higher WUE_p) (Chapter-5 section 5.3.3.4 and 5.3.2.4), greyish leaf colour and visually higher leaf angles. As described earlier, the F_3 generation from genotype $59F_2$(*Ds*+) had

reduced g_s but this effect was lost in F_4 progeny. This could be explained by gene silencing triggered by the aberrant expression of the transgene (Vaucheret et al., 1998) or due to the loss of one of the *Tr-Ds* elements through segregation, responsible for the reduction in the g_s.

Significant reduction in the g_s was observed in genotype $102F_5$ (*Ds*+), this genotype had up to 70% lower g_s with up to 60% higher WUE_p compared to the wild type plants (chapter-6, Table 6-4). However, this genotype did not have any of the issues with seed germination or slow establishment at seedling stage that were present in the sp5 and sp12 lines in which NCED was driven by the Gelvin super promoter (Thompson et al., 2000) and it therefore represents a step forward in producing useful high ABA and high WUE_p plants. However, this genotype had lower net assimilation rate and relative growth rate (RGR) compared to the wild type up to 28 DAG which might contribute to reduced crop yield under well watered conditions. However, under water limited environments it might outperform the wild type; this idea is illustrated in Figure 9-1 which shows that under limited water availability there might be a significant reduction in crop yield in wild type but high ABA genotypes such as $102F_2$(*Ds*+) can conserve the soil water for longer and might not suffer significant yield losses. This genotype could potentially be commercially viable in drier environments after cross pollination to create an F_1 hybrid with high vigour, which might have reduced the WUE_p but improved growth in comparison to the homozygous *Ds* line. It was observed (although data was not recorded) that the genotype $102F_5$(*Ds*+) plants produced large bunches of mature fruit comparable to the wild type plants, suggesting that high ABA did not affect the plant yield, however, this can only be proved once data on the yield is carefully recorded in field or commercial glasshouse environments.

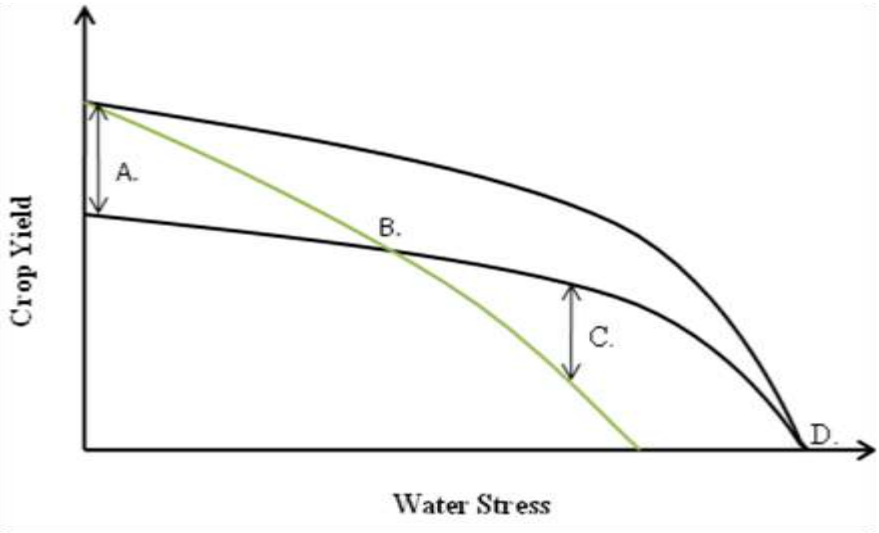

Figure 9.1: A hypothetical representation of the relationship between water stress and crop yield. Arrow 'A' represents the potential yield gap between a wild type (green line) and high ABA genotype (lower black line); point 'B' shows the 'crossover point' where under higher levels of water stress the high ABA genotype surpasses the wild type in crop yield; 'C' represents the potential yield advantage between the high ABA genotype and wild type 'D' illustrates the total loss of yield at very high water stress. The upper black line represents an ideal genotype which can have higher yield under higher levels of water stress.

9.2 Approaches to screen putatively high ABA plants

A rapid but effective screening at an early growth stage can be very useful as more plants with an interesting high ABA phenotype could be selected for further work, giving more insight into how patterns and levels of ABA accumulation influence g_s, WUE_p and plant development. For instance, the infra-red thermal imaging approach should theoretically show the differences between the plants with high and low transpiration rates under uniform light and temperature, as the leaves of the plants with higher transpiration rates should be cooler than those with lower rates (Merlot et al., 2002). However, during this study no differences could be detected under glasshouse or controlled (Weiss room) environment. This could partly be due to too low rates of

absolute transpiration as the set temperature (22 ^{o}C), irradiance (400 μmol m^{-2} s^{-1}) or VPD (0.92 kPa) might not be enough to push the genotypes to their highest transpiration rates. Under such conditions, differences in transpiration would cause only small changes in leaf temperature. For future studies it is suggested to increase the temperature (23- 27^{o}C) and/or increase the VPD (> 3 kPa); this will increase transpiration and any difference in g_s and therefore transpiration would more readily be detected as leaf temperature differences. Similarly, the presence of dry and wet references in each image is crucial and must be standardised (Grant et al., 2006); all leaves being imaged should be under a homogenous radiation environment with a similar angle towards the sun or source of irradiation (Jones, 2004).

The second screening approach was to measure the g_s directly using either infra-red gas analyser (IRGA) or a steady-state Porometer. The IRGA could be used under a range of environmental conditions but taking multiple readings per plant was a challenge. Hence, fewer readings could be carried out by using IRGA in comparison to Porometer. As this work involved screening of a large number of plants, IRGA approach was impractical. Contrary to this the steady-state Porometer is a quick and robust instrument which can be used over a large number of plants. As the stomatal responses are highly dynamic it is crucial to take measurements without much time in between the different genotypes, for example a passing cloud could result in stomatal closure which could confound results. The Weiss controlled environment room facilities played an important role in screening on the basis of g_s because most of the confounding environmental variation could be removed. However, it is worth considering that controlled environment facilities might not be available in all agricultural research

stations especially in the developing world so screening strategies under variable conditions are required.

Visual plant inspection could also be used to screen high ABA plants at a lower cost. For instance, it was observed that the $102F_2$(*Ds*+) plants had interveinal flooding (observed only under low VPD) and higher leaf angle (Chapter-6, Table 6-1) classic symptoms of high ABA tomato plants (Thompson et al., 2007). However, these symptoms might not be present during early growth stages and these symptoms might be confused with leaf mineral deficiency, or maybe induced in wild type plants at high relative humidity. The use of more integrative approaches such as gravimetric water use and carbon isotope discrimination ($\Delta^{13}C$) which are less dependent on the instantaneous environmental conditions can be employed to avoid such ambiguity.

9.2.1 A critical review of plant material and techniques used during the study

As described in Chapter-1, the parent transgenic tomato plants crossed during this study had different genetic backgrounds. The *sAc* element was transformed in tomato cultivar 'Money Maker' and the *Ds* element was transformed into tomato cultivar 'Ailsa Craig'. In the F_2 progeny this could potentially cause variation in traits that would affect WUE_p and g_s. Hence, the present experiments could have been better performed if the *sAc* and *Ds element*s were available in the same genetic background. In order to minimise the effect of variation in the genetic background only the segregating population obtained from heterozygote parents was used during the experiments designed to establish the effect of the *Ds* elements. The parent plant containing the T-DNA-*Ds* element (line 517-1) had more than one copy of the *LeNCED1* transgene, (Figure 5-15). This could have had its own effect on the g_s, however this was tested and

no effect of the T-DNA-*Ds* element was found on the plant g_s (Chapter-5 section 5.4.4). There was increased seed dormancy which could be attributed to the *Ds* element or it might be due to somaclonal variation occurring during the tissue culture.

The gravimetric assessment is a robust technique which provides simultaneous measurement of plant transpiration and its biomass accumulation over a set period of time. However, it is important to note that this technique is laborious and time consuming. It will be useful if the root biomass could also be included in the measurements while performing the gravimetric experiments which will give a better understanding of root:shoot partitioning (Thompson et al., 2007).

During the present study, various important plant traits were studied which could be involved in controlling the WUE_p. For instance, the $[ABA_{xyl}]$ and *LeNCED1* expression and their correlation with the WUE_p which suggests a strong positive correlation between $[ABA_{xyl}]$ and WUE_p. However, the correlation between DW and $[ABA_{bl}]$ during early growth (14 DAG) stages was negative (chapter-6 Figure 6-12). As the plants grew older, the negative correlation diminished (chapter-6 Figure 6-12). Contrary to this, a negative correlation was found between $[ABA_{xyl}]$ and DW at 42 DAG but not at 28 or 56 DAG (Chapter-6 Figure 6-13). Due to time and space limitations, the gravimetric experiments could not be performed on a large scale. Also, it would have been ideal to carry out these experiments in the field conditions under semi-arid environment to evaluate the potential differences in WUE_p and associated effects on yield.

9.3 Compatibility between WUE_p and a genotype's ability to gain biomass

Through the expression of NCED genes, plant ABA content increased (Thompson et al., 2000, Iuchi et al., 2001, Soar et al., 2004, Tung et al., 2008). This resulted in reduced g_s and increased the WUE_p, but this also resulted in reduced DW or plant biomass under well watered conditions (Tung et al., 2008). The observation that reduced g_s results in reduced DW was highlighted previously in cereal crops and through crop simulations by reducing the g_s (Condon et al., 2002, Sinclair, 2005). However, under limited water availability genotype $102F_5$(*Ds*+) might surpass the wild type and be able to produce a harvestable crop, as it is able to maintain higher Ψ_p necessary for growth maintenance. This notion is further strengthened with a recent finding that in *A.thaliana* during early developmental stages the leaf expansion is controlled mainly by C-reserves but in older leaves (4-days old) the leaf expansion was solely driven by the plant water status but not the C-reserves (Pantin et al., 2011). Hence it shows that C-assimilation due to increased g_s is not the sole factor responsible for leaf expansion as highlighted by Condon et al. 2002, Sinclair et al. 2005 and Blum 2005. Leaf expansion can only be achieved if a plant can maintain a positive turgor pressure under water deficit environment.

The antagonistic interaction between ABA and ethylene has improved our understanding in ABA role to help plant growth. For instance under availability of optimum growth conditions including water and RH in ABA mutant genotype (*notabilis)* cannot restore a normal growth (Thompson et al., 2004, Sharp and LeNoble, 2002) but it can start growing normally with the exogenous application of ABA (Sharp and LeNoble, 2002), which interacts with ethylene reducing its effect in excessive

production of reactive oxygen species (ROS) and hence maintains growth (Dante, 2011).

One of the best genotypes observed during the experiments was sp5 because it had high WUE_p without a penalty in long-term DW and also a higher NAR and RGR compared to the wild type and $102F_5$(*Ds*+) plants approximately four-weeks after seed germination (chapter-6 section 6.2.3.3.4 and 6.2.3.3.5). Although genotype $102F_5$(*Ds*+) had a lower final DW compared to sp5 plants, it was observed that both the genotypes $102F_5$(*Ds*+) and sp5 had higher NAR (Chapter-6, Figure 6-5) and RGR (chapter-6, Figure 6-6) compared to the wild type plants at approximately 42 and 56 DAG. The differences between sp5 and $102F_5$ are probably due to the construct design with different promoters ("super-promoter" in case of sp5 and histone H2A in $102F_5$(*Ds*+)), but this might also be due to the difference in the genetic background of the two genotypes because the control Ailsa Craig plants (the genetic background for sp5) were not included in these experiments due to space restrictions. It would have been ideal to compare the effect of the sp5 transgene and the effect of the $102F_5$*Ds element* to their respective parental backgrounds to establish if the difference in growth between sp5 and $102F_5$(Ds+) was really due to the NCED transgenes or whether the different backgrounds made a contribution.

An increased RGR and NAR in sp5 and $102F_5$(*Ds*+) plants might be due to rapid mobilization of starch reserves and its allocation to photosynthetic tissues between 4-8 weeks after seed germination. Seiler et al. (2011) showed a positive correlation between ABA and the mobilization of starch contents in the flag leaves of barley plants stored in the developing seed (Seiler et al., 2011). This might be due to activation of ABA responsive *cis*-elements found in the promoter regions of important starch biosynthesis

genes such as *HvSUS1* and *HvAGP-L1*. Hence, one hypothesis is that ABA may be involved in the mobilization of photosynthates to various rapidly developing plant parts such as seeds, roots and shoots. The starch mobilization in high ABA genotypes or through exogenous application of ABA could be commercially exploited to increase the fruit or grains yield in crops. However, in a similar study, it was recorded that high ABA genotypes such as sp12 and sp5 had higher glucose, fructose and starch contents in the first fully expanded leaves and newly grown roots. So, although this suggests a role of ABA in enhancing carbohydrate supply to the plant, the mechanisms were not further investigated to establish if the increases were balanced by decreases elsewhere, such as in stems or reproductive tissues (Thompson, 2003). However, the significance of the finding that high ABA genotypes (sp5 and $102F_5$(*Ds*+)) had relatively lower carbon partitioning to the stem compared to the wild type plants (Chapter-6 Table 6-3) is highlighted and requires more concentrated effort to evaluate the relationship between ABA and starch remobilization.

More work clearly needs to be done to investigate the relationship between starch mobilization and yield potential of genotype $102F_5$ (Ds+) and sp5, as none of the studies carried out so far involved the assessment of fruit yield potential of this genotype under either reduced water availability or well watered conditions. It is necessary to grow these genotypes under natural environmental conditions (field trials) to assess the real effect of increased ABA content on various agronomic parameters.

9.3.1 Delivering enhanced WUE_p and its prospective role in agriculture:

As the world population has now exceeded seven-billion the pressure on food resources will continue to increase. As agriculture is the major consumer of fresh water

in most parts of the world, with increasing population and aridity, fresh water will soon be a scarce commodity. This could cause a large scale famine, starvation and riots over food and water resulting in economic and political instability especially in the developing parts of the world.

The findings of this study can be useful for commercial crop production as genotype $102F_5$(*Ds*+) might be suited to environments where water availability is severely restricted. The tradeoff between higher WUE_p and lower biomass under optimum conditions (yield potential) may be acceptable depending on the availability and price of water in a particular location. It is also important to clarify the criticism of some researchers that 'reducing g_s to increase WUE_p is not a solution to the problem in drier environment' (Blum, 2009), as the genotype $102F_5$ (*Ds*+) which has no germination issues, can produce some fruit and DW while preserving soil water beyond the reproductive phase, while plants with a higher demand for water might fail altogether in case of rapid use of available water in the root zone, leading to complete crop failure. On the other hand, genotype sp5 can be an excellent candidate to save irrigation water in environments where water shortage is less acute, for example, in Western Europe and some parts of USA. In the case of sp5 where germination and establishment is slowed, robust protocols would be needed to produce suitable seedlings for transplanting into the field. Chemical inhibitors of NCED could be useful to produce rapidly growing seedlings from sp5. One of the commercial uses of the NCED inhibitors could be to prime the seed with these inhibitors so the seed could be used for direct drilling for a uniform germination in the field.

The findings of this study and the previous studies confirm that the manipulation of ABA contents in tomato plants can increase the WUE_p under well

watered conditions by reducing the g_s. However, its dose is critical for plant growth and development at various developmental stages.

As the yield (Y) of a crop variety depends on the genotype's ability to produce biomass (G) and the cultivation environment, a useful expression can be devised i.e.

Y = ⟶ Equation 8-1

But the crop yield is closely related to the crop management (M) practices hence the equation 8-1 can be redefined as;

Y = M ⟶ Equation 8-2

As mentioned in equation 8-2 the crop yield also depends on 'M' and any changes in crop management practices would significantly affect the crop yield. Under the drier environments, it is suggested to use water saving techniques such as deep tilling, mulching and drip irrigation in combination with the use of high WUE_p genotypes such as $102F_5$(Ds+) and sp5.

9.3.2 Other approaches to improve WUE_p through manipulation of ABA

Another mechanism to manipulate the distribution of ABA in plants is by up regulation of ABA transporters (i.e. ABCG25 and ABCG40) (Kuromori et al., 2010, Kang et al., 2010) and receptors (PYR/PYL) (Melcher et al., 2010). The up regulation of receptors could increase the stomatal sensitivity to the existing ABA in the cell, hence restricting the stomatal pore and g_s. One major difference of the manipulating ABA receptors is that effects could be targeted more easily, as receptors act in a cell-autonomous way unlike ABA which is highly mobile in the plant. However, this is not

always an advantage because increasing ABA content could potentially still allow interaction with the normal environmental factors such as VPD, whereas the intervention later in the signaling pathway may short-circuit such regulation.

The roots sense onset of drought in the first instance, and the use of transgenic high ABA rootstocks could be useful in combating drought. As a consequence of drought, the rootstock should produce sufficient quantity of ABA required to reduce the g_s on the scion. This "semi-GM" approach can circumvent the objections made against the production of GM crops as the fruits will be devoid of any foreign DNA material. However, it was observed that high ABA genotype sp5 rootstock cannot supply enough ABA to the scion as the NCED in the rootstock does not have enough carotenoid precursors available in the rootstock. To overcome this issue triple rootstocks over expressing *LePSY1*, *LeBCH2*, and *LeNCED1* were designed to increase the ABA biosynthesis to affect the g_s in the wild type scion (Smeeton, 2010).

The exploitation of natural allelic variation is a possible non-GM approach.

wild tomato species (*Solanum galapagense* and *Solanum neorickii*) and its introgression in the cultivated tomato genetic background to introgress natural alleles that could improve WUE_p

Quantitative Trait Loci' (QTL) can be used to analyse the natural genetic variation in different crop species. The observation that the high WUE_p correlates better with low transpiration rather than high C-assimilation (Yoo et al., 2009), implies that plants have evolved through control of transpiration. QTL for transpiration efficiency have been identified and in one case the gene underlying a QTL has been identified (ERECTA) *A.thaliana* (Masle et al., 2005). However, more work needs to be done in order to identify the genes present in a QTL which are responsible for a particular phenotype in

commercial crops such as wheat, rice and soybean. Association mapping is also a strong complementary approach in order to discover new loci that influence WUE_p, or to confirm and increase the accuracy of known QTL. With the availability of whole genome sequence accompanied by better and efficient sequencing techniques, more genes controlling a particular QTL should soon be identified in the commercial crops for high WUE_p.

9.4 Agronomic and commercial importance of the findings in this study

One of the key criticisms of genetically modified plants is the presence of plasmid DNA required to select transgenic plants (e.g. the nptII gene within T-DNA). The advantage of the two-way transposon based system is that it allows for the possibility of removal of T-DNA by backcrossing with the wild type plants provided that the *Tr-Ds* integration site is not too closely linked to the original T-DNA. This allows the removal of the kanamycin resistance gene that has been controversial in terms of GM acceptability by consumers and regulators. Achieving a T-DNA-free genetic background by using *sAc/Ds* elements and the availability of whole genome sequence in tomato makes it possible to determine the exact position of the *Tr-Ds* elements, e.g. by next generation sequencing. Multiple *Tr-Ds* elements could then be segregated by further rounds of backcrossing with the wild type plants which could result in plants with further variation in the high ABA phenotype.

As mentioned in Chapter-7, (section 7.5), genotype $102F_2$(Ds+) was backcrossed with the wild type (Ailsa Craig $Tm2^a$) to segregate the T-DNA from *Tr-Ds* elements which was accomplished successfully. These plants have also been cross pollinated with

sAc plants to reactivate the *Tr-Ds* element. This F_1 progeny (*Tr-Ds* × *sAc*) can be used to produce a multitude of F_2 plants with a variety of [ABA] but without T-DNA backbone.

This work has shown that NCED inhibitors can improve the germination of seed in situations where increased ABA levels are the cause of reduced rates of germination. The preliminary results of this study showed that the hydroxamic acid D4 can improve the seed germination in situations where increased ABA levels are the cause of reduced rates of germination. It might also improve plant growth, though not significantly in this study. It will be interesting to know if applications of higher concentration (> 2mM) of this chemical improve the early growth rate of slow-to-establish genotypes such as sp5 and sp12. The hydroxamic acids used during the present study can be very useful in crops with mild form of secondary dormancy mediated by ABA. As these chemicals specifically target NCED gene, a key regulatory enzyme in ABA biosynthesis, they can be used to alleviate dormancy in potato, onion and thermo dormancy (induced by high temperature). However, to improve its absorption and translocation, surfactants such as polyethoxylated amine (a wetting agent) could be used (Ahle, 1985). Additional research is required to develop more potent and effective NCED inhibitors perhaps using D4 as the lead compound to develop chemical variants.

On the basis of these findings, more work is suggested which includes the effect of increased ABA content on plant reproductive maturity and yield. Because tomato fruit is of commercial importance, a study should be dedicated to assess the role of ABA on tomato fruit yield. As transpiration helps in plant cooling, a cap on g_s might increase the leaf temperature under hot environmental conditions which could cause limitation to the plant photosynthetic apparatus resulting in yield losses. Therefore it will also be

desirable to evaluate the effect of high temperature and high irradiance on the performance of high ABA genotypes in the field.

Chapter-10

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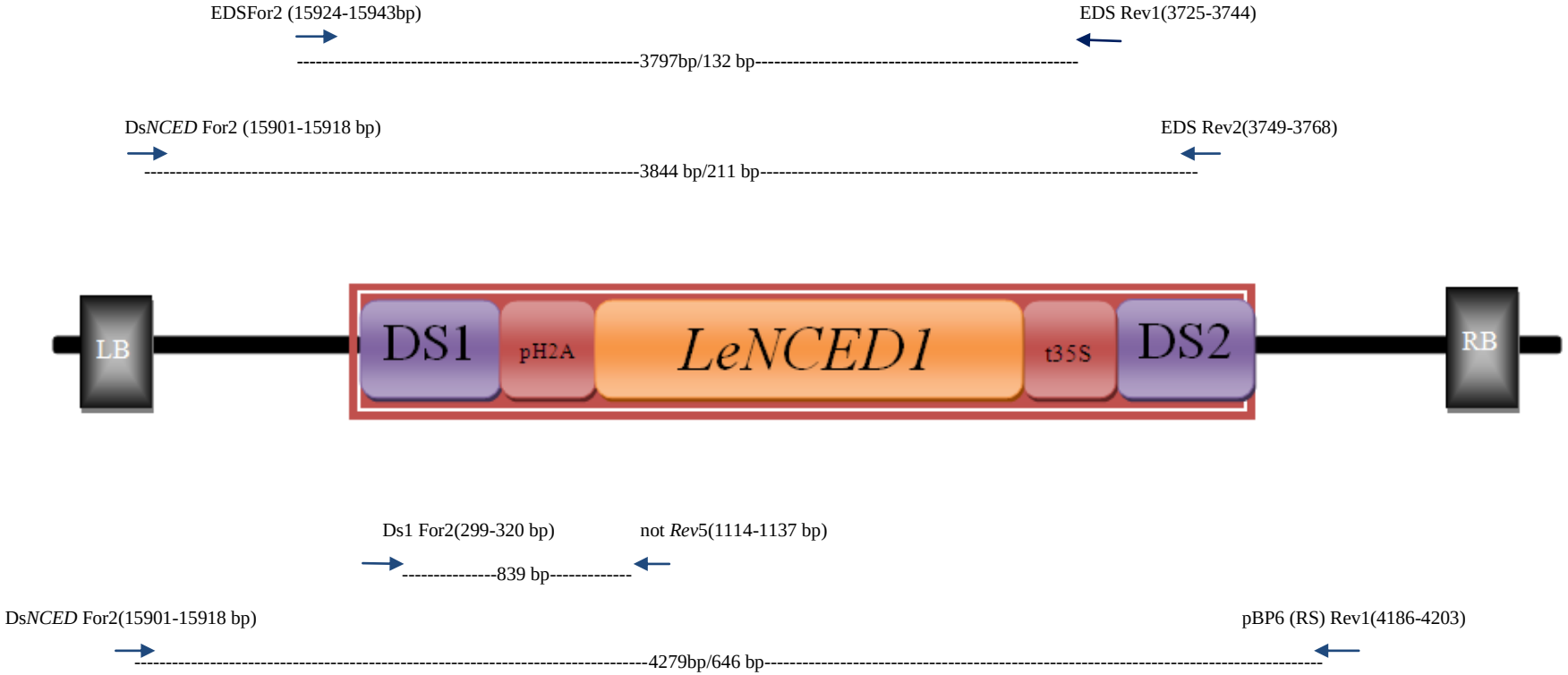
Appendix I

Primers, PCR conditions and predicted sizes of various amplicons of the Plasmid *pBP6*

Primers	Starts at (bp)	End point (bp)	Primer sequence 5' to 3'	PCR conditions	No. of cycles	Predicted amplicon size (bp)	Purpose
Ds1 For2	299	320	CCCAGCCCATGTAAGAAATACC	95 °C for 2 min 95 °C for 30 S 54 °C for 30 S 72 °C for 1 min	25	839	To amplify a part of Ds element from the gene of interest
not Rev5	1114	1137	CTGCACTAAATTCCACTTGGAAGTTG	72 °C for 5 min			
DsNCED For2	15901	15918	CCGGCTCGTATGTTGTGT	94 °C for 3 min 94 °C for 10 S 57 °C for 1 min 72 °C for 1 min 68 °C for 4 min	35	4279 or 646	To amplify the complete gene of interest, including both Ds1 and Ds2 elements
pBP6(RS)Rev1	4186	4203	GTTGGTGCAATTTGCCTG				
DsNCED For2	15901	15918	CCGGCTCGTATGTTGTGT	94 °C for 3 min 94 °C for 10 S 57 °C for 1 min 72 °C for 1 min 68 °C for 4 min	30	3844 or 211	To amplify the complete gene of interest and Empty Donor Site (EDS)
EDS Rev2	3749	3768	AGCTGGCGTAATAGCGAAGA				
EDS For2	15924	15943	TGTGAGCGGATAACAATTTC	94 °C for 3 min 94 °C for 10 S 57 °C for 1 min 68 °C for 4 min 72 °C for 5 min	25	132 or 3797	Nested set of Primers to amplify the EDS for sequencing purposes
EDS Rev1	3725	3744	CTTGCAGCACATCCCCCTTT				
Ds-T-DNA-For1	15876	15895	CACCCCAGGCTTTACACTTT	95 °C for 2 min 95 °C for 30 S 54 °C for 30 S 72 °C for 1 min 72 °C for 5 min	30	399	To amplify T-DNA-Ds element for selection of T-DNA free genotypes
Ds-T-DNA-Rev1	280	299	CCTCAGTGGTTATGGATGGG				

Appendix-II

T-DNA-Ds diagram illustrating the positions of different primers used during the experiment

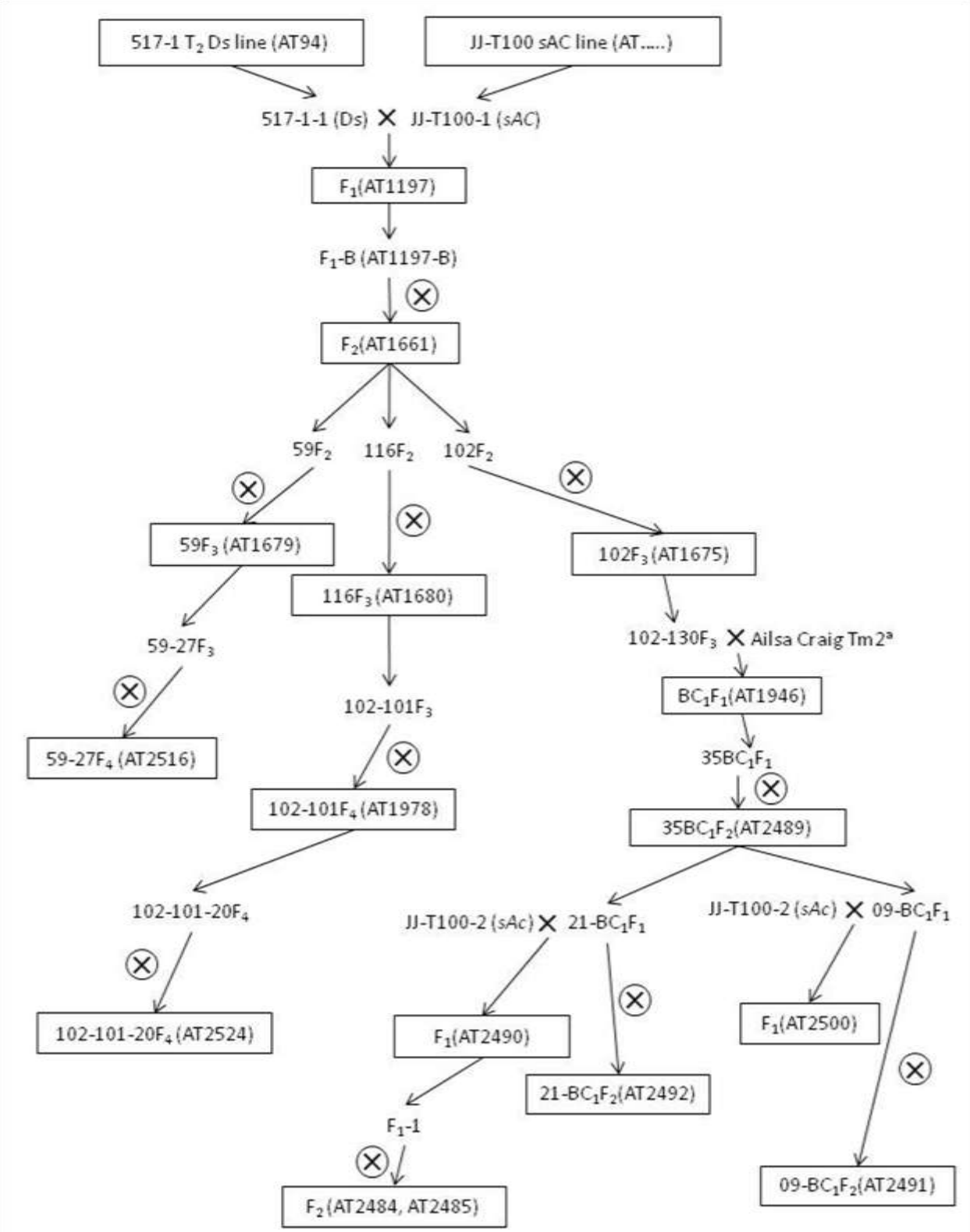


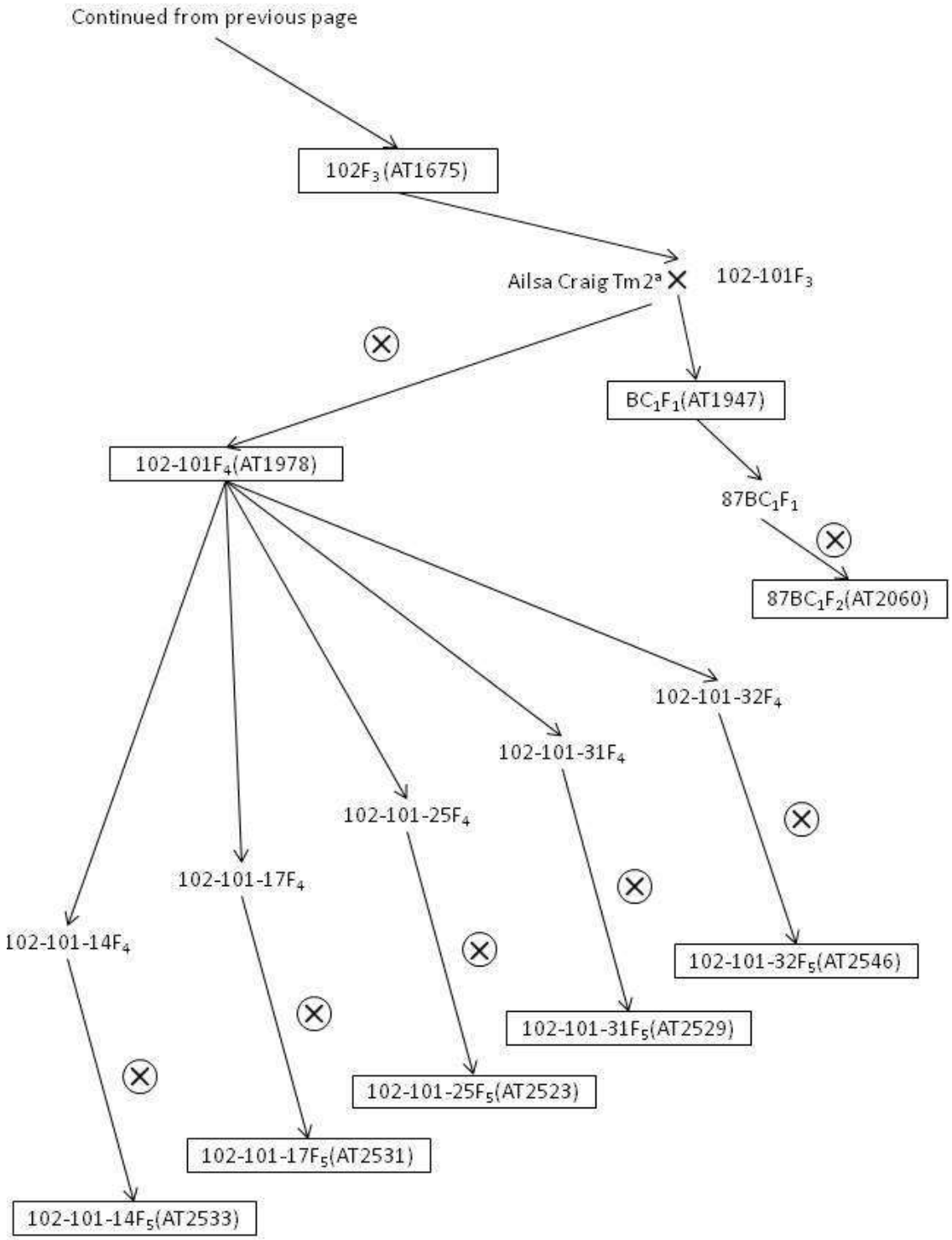
A schematic diagram (not to scale) showing various components present in the Ds1:pH2A::*LeNCED1*::t35s:Ds2 construct. LB and RB represent left and right borders, respectively. nptII gene is the kanamycin resistance gene. DS1 and DS2 are dissociation element 1&2 respectively. pH2A is the Histone promoter, t35S is the terminator sequence from CMV. *LeNCED1* is the tomato 9-cis-epoxy carotenoid dioxygenase gene.

*Nested set of Primers to amplify Empty Donor Site (EDS)

Direct Primer
Complementary primer ------ Expected PCR product

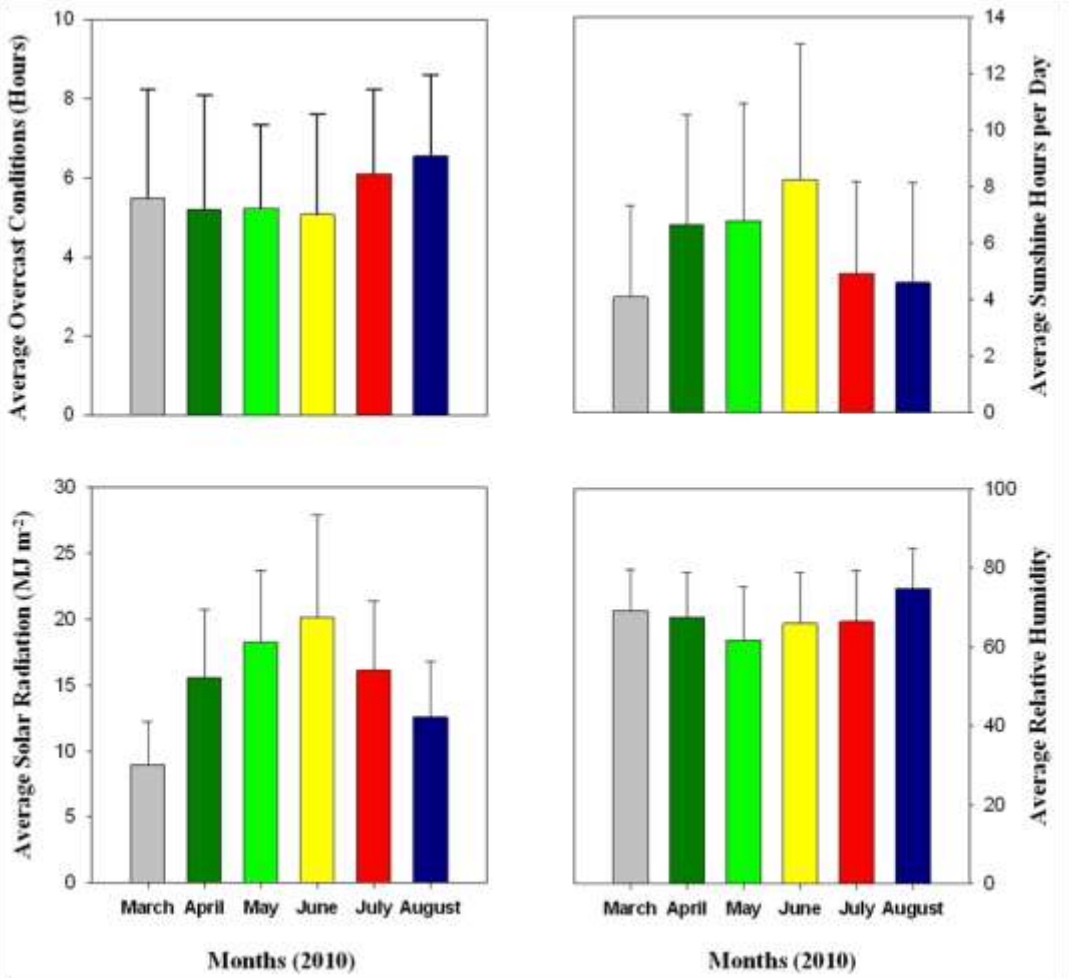
Appendix-III





Appendix-III. A pedigree chart showing the seed bulking in the selected genotypes. Numbers present outside the boxes represent an individual plant; a cross sign '×' shows plant cross pollination; a cross '×' present in a circle shows self pollination. The numbers present in the boxes shows the seed accession number.

Appendix-IV



Appendix-IV. Average climatic conditions recorded in the glasshouse during 2010. Data was collected with the help of a quantum sensor attached to a data logger (Delta T devices, Cambridge, UK). Sensors were placed in the same glasshouse where the experiments were conducted.